

519, 135

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
15 January 2004 (15.01.2004)

PCT

(10) International Publication Number
WO 2004/005555 A1

- (51) International Patent Classification?: **C12Q 001/60**, C12N 015/29
- (74) Agent: **F B RICE & CO**; 139-141 Rathdowne Street, Carlton, Victoria 3053 (AU).
- (21) International Application Number: PCT/AU2003/000854
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (22) International Filing Date: 2 July 2003 (02.07.2003)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data: PS 3339 2 July 2002 (02.07.2002) AU
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
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- Published:
— with international search report
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: METHOD OF PRODUCING PLANTS HAVING ENHANCED TRANSPIRATION EFFICIENCY AND PLANTS PRODUCED THEREFROM

(57) Abstract: The present invention provides methods of selecting plants having modified transpiration efficiency using plant ERECTA gene sequences and nucleic acids linked thereto, and to methods of producing plants having modified transpiration efficiency using isolated plant ERECTA gene sequences, in both traditional plant breeding and genetic engineering approaches. The invention further extends to plants produced by the methods described.

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METHOD OF PRODUCING PLANTS HAVING ENHANCED TRANSPIRATION EFFICIENCY AND PLANTS PRODUCED THEREFROM

FIELD OF THE INVENTION

The present invention relates to the field of plant breeding and the production of
5 genetically engineered plants. More specifically, the invention described herein
provides genes that are capable of enhancing the transpiration efficiency of a plant
when expressed therein. These genes are particularly useful for the production of
plants having enhanced transpiration efficiency, by both traditional plant breeding and
genetic engineering approaches. The invention further extends to plants produced by
10 the methods described herein.

BACKGROUND TO THE INVENTION

1. General

This specification contains nucleotide and amino acid sequence information prepared
using PatentIn Version 3.1, presented herein after the claims. Each nucleotide
15 sequence is identified in the sequence listing by the numeric indicator <210> followed
by the sequence identifier (e.g. <210>1, <210>2, etc). The length and type of sequence
(DNA, protein (PRT), etc), and source organism for each nucleotide sequence, are
indicated by information provided in the numeric indicator fields <211>, <212> and
<213>, respectively. Nucleotide sequences referred to in the specification are defined
20 by the term "SEQ ID NO:", followed by the sequence identifier (eg. SEQ ID NO: 1
refers to the sequence in the sequence listing designated as <400>1).

The designation of nucleotide residues referred to herein are those recommended by the
IUPAC-IUB Biochemical Nomenclature Commission, wherein A represents Adenine,
25 C represents Cytosine, G represents Guanine, T represents thymine, Y represents a
pyrimidine residue, R represents a purine residue, M represents Adenine or Cytosine, K
represents Guanine or Thymine, S represents Guanine or Cytosine, W represents
Adenine or Thymine, H represents a nucleotide other than Guanine, B represents a
nucleotide other than Adenine, V represents a nucleotide other than Thymine, D
30 represents a nucleotide other than Cytosine and N represents any nucleotide residue.

As used herein the term "derived from" shall be taken to indicate that a specified integer is obtained from a particular source albeit not necessarily directly from that source.

5

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated step or element or integer or group of steps or elements or integers but not the exclusion of any other step or element or integer or group of
10 elements or integers.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The
15 invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

The present invention is not to be limited in scope by the specific embodiments
20 described herein, which are intended for the purposes of exemplification only. Functionally equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

2. Description of the related art

It is well known that virtually all plants require a certain quantity of water for proper growth and development, because CO₂ fixation and photosynthate assimilation by
25 plants cost water. A significant quantity of water absorbed by plants from the soil returns to the atmosphere *via* plant transpiration.

Transpiration efficiency is a measure of the amount of dry matter produced by a plant per unit of water transpired, or, in other words, carbon gain relative to water lost through transpiration.

- 5 For plants having low transpiration efficiency, or when water is in short supply, the loss of water through transpiration can limit key metabolic processes associated with plant growth and development. For example, during drought, or when plants having low transpiration efficiency are grown in arid and semi-arid environments, plant productivity as determined by dry matter production or photosynthetic rate, is
- 10 considerably reduced. Accordingly, the production of plants having enhanced water use efficiency or transpiration efficiency is highly desirable for their adaptation to arid or semi-arid conditions, or to enhance their drought resistance.

- The enhancement of water use efficiency or transpiration efficiency by plants is also
- 15 highly desirable in consideration of global climatic change and increasing pressure on world water resources. The inefficient utilization of agricultural water is known to impact adversely upon the supply of navigable water, potable water, and water for industrial or recreational use. Accordingly, the production of plants having enhanced transpiration efficiency is highly desirable for reducing the pressure on these water
- 20 resources. It is also desirable for increasing plant productivity under well-watered conditions.

- By enhancing transpiration efficiency, carbon gain rates are enhanced per unit of water transpired, thereby stimulating plant growth under well-watered conditions, or
- 25 alternatively, under mild or severe drought conditions. This is achieved by enhancing carbon gain more than transpiration rate, or by reducing the amount of water lost at any particular rate of carbon fixation. Those skilled in the art also consider that for a given growth rate plants having enhanced transpiration efficiency dry out soils more slowly, and use less water, than less efficient near-isogenic plants.

Several chemical as well as environmental pre-treatments have been described for enhancing the ability of plant seedlings to survive drought, either by reducing transpiration or by reducing the amount of water that is actually lost to the atmosphere.

- 5 Known environmental treatments largely involve the use of physical barriers. Whilst placing a physical barrier over plant stomata is known to reduce water loss via transpiration, the procedure is not always desirable or practicable for field-grown crops. For example, physical barriers over plant stomata may inhibit certain gas-exchange processes of the plant. It is more desirable to enhance actual transpiration efficiency or
10 water use efficiency of the plant through manipulation of intrinsic plant function.

Chemical agents are typically the so-called "anti-transpirant" or "anti-desiccant" agents, both of which are applied to the leaves. Anti-transpirants are typically films or metabolic anti-transpirants.

15

- These products form a film on leaves, thereby either blocking stomatal pores, or coating leaf epidermal cells with a water-proof film. Typical film anti-transpirants include waxes, wax-oil emulsions, higher alcohols, silicones, plastics, latexes and resins. For example, Elmore, United States Patent No. 4,645,682 disclosed an anti-
20 transpirant consisting of an aqueous paste wax; Cushman *et al.*, United States Patent Nos. 3,791,839 and 3,847,641 also disclosed wax emulsions for controlling transpiration in plants; and Petrucco *et al.*, United States Patent No. 3,826,671, disclosed a polymer composition said to be effective for controlling transpiration in plants.

25

Metabolic anti-transpirants generally close stomata, thereby reducing the rate of transpiration. Typical metabolic anti-transpirants include succinic acids, phenylmercuric acetate, hydroxysulfonates, the herbicide atrazine, sodium azide, and phenylhydrazones, as well as carbon cyanide.

30

Compounds having plant growth regulator activity have also been shown to be useful for reducing transpiration. For example, Bliesner *et al.*, United States Patent No. 4,671,816, disclosed an acetylene compound, said to possess utility for regulating plant growth, whilst Kuznetsov *et al.* (Russian Patent No. SU 1,282,492; ., Russian Patent Application No. SU 1,253,559-A1), and Smirnov *et al.* (Russian Patent No. SU 1,098,934) disclosed the use of derivatives of 2-methyl-5-hydroxybenzimidazole, and the chloride or bromide salts thereof, as anti-transpirant growth regulators. Vichnevetskaia (USSN 5,589,437 issued December 31, 1996) also describe hydroxybenzimidazole derivatives for enhancing the drought resistance of plants by reducing transpiration. Schulz *et al.*, United States Patent No. 4,943,315, also disclosed formulations comprising an acetylene and a phenylbenzylurea compound, for reducing transpiration in plants and/or for avoiding impairment to plants caused by heat and dry conditions. Abscissic acid has also been shown to reduce or suppress transpiration in plants (eg. *Helv. Chim. Acta*, 71, 931, 1988; *J. Org. Chem.*, 54, 681, 1989; and Japanese Patent Publication No. 184,966/1991).

Metabolic anti-transpirants are costly to produce and often exhibit phytotoxic effects or inhibit plant growth (Kozlowski (1979), *In: Tree Growth and Environmental Stresses* (Univ. of Washington Press, Seattle and London)), and are not practically used.

20

Recent studies have examined alternative methods for enhancing transpiration efficiency, particularly breeding approaches to select lines that grow more efficiently under mild drought conditions. Carbon isotope discrimination has been used to identify *Arabidopsis* ecotypes with contrasted transpiration efficiencies (Masle *et al.*, *In: Stable isotopes and plant carbon-water relations*, Acad. Press, Physiol. Ser., pp371-386, 1993) and to assist conventional breeding of new plant varieties in a number of species (Hall *et al.*, *Plant Breeding Reviews* 4, 81-113, 1994) including rice (Farquhar *et al.*, *In: Breaking the Yield Barrier*, ed KG Cassman, IRRI, 95, 101) and most recently wheat (Rebetzke *et al.* *Crop Science* 42:739-745, 2002).

30

No single gene has been identified as being capable of enhancing transpiration efficiency when expressed *in planta*. Transpiration efficiency may well be multigenic. As a consequence, the genes and signalling pathways that regulate the photosynthetic and/or stomatal components of the transpiration efficiency mechanism in plants have
5 not been identified or characterized.

Moreover, notwithstanding that the effect of down-regulating expression of the *Rubisco* gene, or mutation in genes involved in abscisic acid (eg. *aba*, *abi*), are known to modify transpiration efficiency to some extent through stomatal closure, the consequence of
10 such modifications is not necessarily specific, resulting in pleiotropic effects.

Arabidopsis thaliana ecotype Landsberg *erecta* (*L-er1*) is one of the most popular ecotypes and is used widely for both molecular and genetic studies. It harbors the *er1* mutation, which confers a compact inflorescence, blunt fruits, and short petioles. There
15 are a number of *erecta* mutant alleles. Phenotypic characterization of the mutant alleles suggests a role for the wild type *ER* gene in regulating plant morphogenesis, particularly the shapes of organs that originate from the shoot apical meristem. Torii *et al.*, *The Plant Cell* 8, 735, 1996, showed that the *ER* gene encodes a putative receptor protein kinase comprising a cytoplasmic protein kinase catalytic domain, a
20 transmembrane region, and an extracellular domain consisting of leucine-rich repeats, which are thought to interact with other macromolecules.

SUMMARY OF THE INVENTION

In work leading up to the present invention, the inventors sought to elucidate the
25 specific genetic determinants of plant transpiration efficiency. In plants, the development of molecular genetic markers, such as, for example, genetic markers that map to a region of the genome of a crop plant, such as, for example, a region of the rice genome, maize genome, barley genome, sorghum genome, or wheat genome, or a region of the tomato genome or of any Brassicaceae, assists in the production of plants
30 having enhanced transpiration efficiency (Edwards *et al.*, *Genetics* 116, 113 -125, 1987; Paterson *et al.*, *Nature* 335, 721-726, 1988).

The present inventors identified a locus that is linked to the genetic variation in transpiration efficiency in plants. To elucidate a locus associated with the transpiration efficiency of plants, the inventors established experimental conditions and sampling procedures to determine the contribution to total transpiration efficiency of the factors influencing this phenotype, and, more particularly, the genetic contribution to the total variation in transpiration efficiency. Factors influencing transpiration efficiency include, for example, genotype of the plant, environment (eg. temperature, light, humidity, boundary layer around the leaves, root growth conditions), development (eg. age and/or stage and/or posture of plants that modify gas exchange and/or carbon metabolism), and seed-specific factors (Masle et al. 1993, op. cit). The screens developed by the inventors were also used to survey mutant and wild type populations for variations in transpiration efficiency and to identify ecotypes having contrasting transpiration efficiencies including the parental lines that had been used by Lister and Dean (1993). The transpiration efficiencies of the members of Lister and Dean's (1993) Recombinant Inbred Line (RIL) mapping population were then determined, and linkage analyses were performed against genetic markers to determine the chromosome regions that are linked to genetic variation in transpiration efficiency, thereby identifying a locus conditioning transpiration efficiency. Complementation tests, wherein plants were transformed with a wild-type allele at this locus confirmed the functionality of the allele in determining a transpiration efficiency phenotype.

In one exemplified embodiment of the invention, there is provided a locus associated with transpiration efficiency of *A. thaliana*, such as, for example the *ERECTA* locus on *A. thaliana* chromosome 2, or a hybridization probe which maps to the region between about 46cM and about 50.7cM on chromosome 2 of *A. thaliana*. In further exemplified embodiments, the inventors identified additional *ERECTA* alleles or *erecta* alleles in *A. thaliana*, rice, sorghum, wheat and maize which are structurally related to this primary *A. thaliana* *ERECTA* or *erecta* allele. Based upon the large number of *ERECTA/erecta* alleles described herein, the present invention clearly extends to any homologs of the *A. thaliana* *ERECTA* locus from other plant species to those specifically exemplified, and particularly when those homologs are identified using the methods described herein.

Accordingly, one aspect of the invention provides a genetic marker or locus associated with the genetic variation in transpiration efficiency of a plant, wherein said locus comprises a nucleotide sequence linked genetically to an *ERECTA* locus in the genome of the plant. The locus or genetic marker is useful for determining transpiration efficiency of a plant.

As used herein, the terms "genetically linked" and "map to" shall be taken to refer to a sufficient genetic proximity between a linked nucleic acid comprising a gene, allele, marker or other nucleotide sequence and nucleic acid comprising all or part of an *ERECTA* locus to permit said linked nucleic acid to be useful for determining the presence of a particular allele of said *ERECTA* locus in the genome of a plant. Those skilled in the art will be aware that for such linked nucleic acid to be used in this manner, it must be sufficiently close to said locus not to be in linkage disequilibrium or to have a high recombination frequency between said linked nucleic acid and said locus. Preferably, the linked nucleic acid and the locus are less than about 25cM apart, more preferably less than about 10cM apart, even more preferably less than about 5cM apart, still even more preferably less than about 3cM apart and still even more preferably less than about 1cM apart.

20

In a preferred embodiment the present invention provides an isolated nucleic acid associated with the genetic variation in transpiration efficiency of a plant, said nucleic acid comprising a nucleotide sequence selected from the group consisting of:

- (a) the sequence of an *ERECTA* genomic gene or the 5'-UTR or 3'-UTR or protein-encoding region or an intron region thereof;
- (b) the sequence of an allelic variant of (a) or the 5'-UTR or 3'-UTR or protein-encoding region or an intron region of said allelic variant;
- (c) the sequence of a fragment of (a) or (b) that hybridizes specifically to nucleic acid (eg., RNA or DNA) from a plant under at least low stringency hybridization conditions; and
- (d) a sequence that is complementary to (a) or (b) or (c).

In a particularly preferred embodiment, the present invention provides an isolated *ERECTA* gene from wheat comprising a nucleotide sequence selected from the group consisting of:

- 5 (i) the sequence set forth in SEQ ID NO: 19;
- (ii) a sequence encoding the amino acid sequence set forth in SEQ ID NO: 20; and
- (iii) a sequence that is complementary to (i) or (ii).

In an alternative embodiment, the present invention provides an isolated *ERECTA* gene
10 from maize comprising a nucleotide sequence selected from the group consisting of:

- (i) the sequence set forth in SEQ ID NO: 44;
- (ii) a sequence encoding the amino acid sequence set forth in SEQ ID NO: 45; and
- (iii) a sequence that is complementary to (i) or (ii).

15 In another alternative embodiment, the present invention provides an isolated *ERECTA* gene from rice comprising a nucleotide sequence selected from the group consisting of:

- (i) the sequence set forth in SEQ ID NO: 3;
- (ii) a sequence encoding the amino acid sequence set forth in SEQ ID NO: 4; and
- (iii) a sequence that is complementary to (i) or (ii).

20

In another alternative embodiment, the present invention provides an isolated *ERECTA* gene from *A. thaliana* comprising a nucleotide sequence selected from the group consisting of:

- (i) the sequence set forth in SEQ ID NO: 1;
- 25 (ii) a sequence encoding the amino acid sequence set forth in SEQ ID NO: 2; and
- (iv) a sequence that is complementary to (i) or (ii).

In another alternative embodiment, the present invention provides an isolated *ERECTA* gene from *A. thaliana* comprising a nucleotide sequence selected from the group
30 consisting of:

- (i) the sequence set forth in SEQ ID NO: 7;

- (ii) a sequence encoding the amino acid sequence set forth in SEQ ID NO: 8; and
- (v) a sequence that is complementary to (i) or (ii).

In another alternative embodiment, the present invention provides an isolated *ERECTA* gene from *A. thaliana* comprising a nucleotide sequence selected from the group consisting of:

- (i) the sequence set forth in SEQ ID NO: 9;
- (ii) a sequence encoding the amino acid sequence set forth in SEQ ID NO: 10; and
- (vi) a sequence that is complementary to (i) or (ii).

10

In yet another alternative embodiment, the present invention provides an isolated *ERECTA* gene from sorghum comprising a nucleotide sequence selected from the group consisting of:

- (i) the sequence set forth in SEQ ID NO: 5;
- 15 (ii) a sequence encoding the amino acid sequence set forth in SEQ ID NO: 6; and
- (vii) a sequence that is complementary to (i) or (ii).

Notwithstanding that an *ERECTA* or *erecta* structural gene or genomic gene or the protein encoding region thereof is particularly useful for breeding and/or mapping purposes, this aspect of the present invention is not to be limited to the *ERECTA* or *erecta* structural or genomic gene or the protein-encoding region thereof. As exemplified herein, the primary *A. thaliana* *ERECTA* locus can be determined using any linked nucleic acid that maps to a region in the chromosome at a genetic distance of up to about 3cM from the *ERECTA* or *erecta* allele. The skilled artisan will readily be able to utilize similar probes to identify linkage to an *ERECTA* or *erecta* allele in any other plant species, based upon the teaching provided herein that the *ERECTA* or *erecta* allele is linked to the transpiration efficiency phenotype of plants.

Preferably, all or part of the locus associated with the transpiration efficiency phenotype in a plant (ie., nucleic acid genetically linked to the *ERECTA* or *erecta* structural or genomic gene) is provided as recombinant or isolated nucleic acid, such

as, for example, in the form of a gene construct (eg. a recombinant plasmid or cosmid), to facilitate germplasm screening.

The *ERECTA* locus or a gene that is linked to the *ERECTA* locus is particularly useful in a breeding program, to predict the transpiration efficiency of a plant, or alternatively, as a selective breeding marker to select plants having enhanced transpiration efficiency. Once mapped, marker-assisted selection (MAS) is used to introduce the *ERECTA* locus or markers linked thereto into a wide variety of populations. MAS has the advantage of reducing the breeding population size required, and the need for continuous recurrent testing of progeny, and the time required to develop a superior line.

Accordingly, a further aspect of the present invention provides a method of selecting a plant having enhanced transpiration efficiency, comprising detecting a genetic marker for transpiration efficiency which marker comprises a nucleotide sequence linked genetically to an *ERECTA* locus in the genome of the plant and selecting a plant that comprises or expresses the genetic marker, preferably wherein the genetic marker comprises an *ERECTA* allele or *erecta* allele, or a protein-encoding portion thereof, or alternatively, wherein the genetic marker comprises a nucleotide sequence having at least about 55% overall sequence identity to at least about 20 nucleotides in length of any one of SEQ ID Nos: 1, 3, 5, 7, 9, 11 to 19 or 21 to 44 or a complementary sequence thereto, including a nucleotide sequence selected from the group consisting of:

- (a) a sequence having at least about 55% identity to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44;

- (b) a sequence encoding an amino acid sequence having at least about 55% identity to an amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 20 and SEQ ID NO: 45; and
- 5 (c) a sequence complementary to (a) or (b).

In an alternative embodiment, the invention provides a method of selecting a plant having enhanced transpiration efficiency, comprising:

- 10 (a) screening mutant or near-isogenic or recombinant inbred lines of plants to segregate alleles at an *ERECTA* locus;
- (b) identifying a polymorphic marker linked to said *ERECTA* locus; and
- (c) selecting a plant that comprises or expresses the marker.

The data exemplified herein for *A. thaliana* or rice can clearly be extrapolated to other
15 plant species. For example, the evidence provided herein for the role of the *A. thaliana* *ERECTA* allele in determining the transpiration efficiency phenotype in those plant species has permitted the elucidation of a wide range of homologous *ERECTA* alleles in other plant species, in particular wheat, rice, sorghum and maize, that are also likely to determine the transpiration efficiency phenotype in those plants. In accordance with
20 this embodiment, the present invention provides a method of selecting a plant having enhanced transpiration efficiency, comprising selecting a plant that comprises or expresses a functionally equivalent homolog of a protein-encoding region of the *ERECTA* gene of *A. thaliana*, maize, wheat, sorghum or rice.

- 25 In a preferred embodiment, the invention provides a method of selecting a plant having enhanced transpiration efficiency, comprising:
 - (a) identifying a locus on the Arabidopsis chromosome 2 (46-50.7 cM) or rice chromosome 6 associated with genetic variation in transpiration efficiency in a plant;

- (b) identifying nucleic acid in a different plant species that comprises a nucleotide sequence having at least about 55% identity to the sequence of the locus at (a); and
- (c) selecting a plant that comprises or expresses the identified nucleic acid at (b).

5

In a further preferred embodiment, this aspect of the invention provides a method of selecting a plant having enhanced transpiration efficiency, comprising:

- (a) identifying a locus on the Arabidopsis chromosome 2 (46-50.7 cM) or rice chromosome 6 associated with genetic variation in transpiration efficiency in a
10 plant;
- (b) determining the nucleotide sequence of the identified locus;
- (c) identifying nucleic acid of a plant species other than *A. thaliana* or rice that comprises a nucleotide sequence having at least about 55% identity to the sequence of the locus at (a); and
- 15 (d) selecting a plant that comprises or expresses the identified nucleic acid at (b).

Preferably, the selected plant according to any one or more of the preceding embodiments is *Arabidopsis thaliana*, rice, sorghum, wheat or maize, however other species are not excluded.

20

Preferably, the subject selection method comprises linking the transpiration efficiency phenotype of the plant to the expression of the marker in the plant, or alternatively, linking a structural polymorphism in DNA to a transpiration efficiency phenotype in the plant, eg., by a process comprising detecting a restriction fragment length
25 polymorphism (RFLP), amplified fragment length polymorphism (AFLP), single strand chain polymorphism (SSCP) or microsatellite analysis. As will be known to the skilled artisan, a nucleic acid probe or primer of at least about 20 nucleotides in length from any one of SEQ ID Nos: 1, 3, 5, 7, 9, 11 to 19 or 21 to 44 or a complementary sequence thereto can be hybridized to genomic DNA from the plant, and the hybridization
30 detected using a detection means, thereby identifying the polymorphism.

It is clearly preferred that the selected plant has enhanced transpiration efficiency compared to a near-isogenic plant that does not comprise or express the genetic marker.

As exemplified herein, the inventors also identified specific genes or alleles that are
5 linked to the *ERECTA* locus of *A. thaliana*, and rice and determined the transpiration
efficiencies of those plants. More particularly, the transpiration efficiencies of near-
isogenic lines, each carrying a mutation within an *ERECTA* locus, and a correlation
between transpiration efficiency phenotype and *ERECTA* expression or gene copy
number are determined, thereby providing the genetic contribution of genes or alleles at
10 the *ERECTA* locus to transpiration efficiency. This analysis permits an assessment of
the genetic contribution of particular alleles to transpiration efficiency, thereby
determining allelic variants that are linked to a particular transpiration efficiency.
Thus, the elucidation of the *ERECTA* locus for transpiration efficiency in plants
facilitates the fine mapping and determination of allelic variants that modulate
15 transpiration efficiency. The methods described herein can be applied to an assessment
of the contribution of specific alleles to the transpiration efficiency phenotype for any
plant species that is amenable to mutagenesis such as, for example, by transposon
mutagenesis, irradiation, or chemical means. As will be known to the skilled artisan
many crop species, such as, maize, wheat, and rice, are amenable to such mutagenesis.

20

Accordingly, a third aspect of the invention provides a method of identifying a gene
that determines the transpiration efficiency of a plant comprising:

- (a) identifying a locus associated with genetic variation in transpiration efficiency in
a plant;
- 25 (b) identifying a gene or allele that is linked to said locus, wherein said gene or
allele is a candidate gene or allele for determining the transpiration efficiency of
a plant; and
- (c) determining the transpiration efficiencies of a panel of plants, wherein not all
members of said panel comprise or express said gene or allele, and wherein
30 variation in transpiration efficiency between the members of said panel indicates
that said gene is involved in determining transpiration efficiency.

In another embodiment, the method comprises:

- (a) identifying a locus associated with genetic variation in transpiration efficiency in a plant;
- 5 (b) identifying multiple alleles of a gene that is linked to said locus, wherein said gene is a candidate gene involved for determining the transpiration efficiency of a plant; and
- (c) determining the transpiration efficiencies of a panel of plants, wherein each member of said panel comprises, and preferably expresses, at least one of said
10 multiple alleles, wherein variation in transpiration efficiency between the members of said panel indicates that said gene is involved in determining transpiration efficiency.

Preferably, the identified gene or allele identified by the method described in the
15 preceding paragraph is an *ERECTA* allele, or an *erecta* allele, from a plant selected from the group consisting of *A. thaliana*, sorghum, rice, maize and wheat, or a homolog thereof.

The identified gene or allele, including any homologs from a plant other than *A.*
20 *thaliana*, such as, for example, the wild-type *ERECTA* allele or a homolog thereof, is useful for the production of novel plants. Such plants are produced, for example, using recombinant techniques, or traditional plant breeding approaches such as introgression.

Accordingly, a still further aspect of the present invention provides a method of
25 modulating (i.e., enhancing or reducing) the transpiration efficiency of a plant comprising ectopically expressing in a plant an isolated *ERECTA* gene or an allelic variant thereof or the protein-encoding region of said *ERECTA* gene or said allelic variant. In a particularly preferred embodiment, the invention provides a method of enhancing the transpiration efficiency of a plant comprising introgressing into said
30 plant a nucleic acid comprising a nucleotide sequence that is homologous to a protein-

encoding region of a gene of *A. thaliana* that maps to the *ERECTA* locus on chromosome 2.

A further embodiment of the invention provides a method of modulating the transpiration efficiency of a plant comprising introducing (eg., by classical breeding, introgression or recombinant means), and preferably expressing therein, an isolated *ERECTA* gene or an allelic variant thereof or the protein-encoding region thereof to a plant and selecting a plant having a different transpiration efficiency compared to a near-isogenic plant that does not comprise the introduced *ERECTA* gene or allelic variant or protein-encoding region. Preferably, the *ERECTA* gene or allelic variant or protein-encoding region comprises a nucleotide sequence selected from the group consisting of:

- (a) a sequence having at least about 55% identity to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43 and SEQ ID NO: 44; and
- (b) a sequence encoding an amino acid sequence having at least about 55% identity to an amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 20 and SEQ ID NO: 45.

The plant into which the gene etc is introduced is preferably selected from the group consisting of *Arabidopsis thaliana*, rice, sorghum, wheat and maize. As will be apparent from the present disclosure, the transpiration efficiency is enhanced as a consequence of the ectopic expression of an *ERECTA* allele or the protein-encoding

region thereof in the plant. In contrast, the transpiration efficiency is reduced as a consequence of reduced expression of an *ERECTA* allele in the plant (eg., by expression of antisense RNA or RNAi or other inhibitory RNA).

- 5 A further aspect of the invention provides for the use of an isolated *ERECTA* gene or an allelic variant thereof or the protein-encoding region of said *ERECTA* gene or said allelic variant in the preparation of a gene construct for modulating (ie., enhancing or reducing) the transpiration efficiency of a plant. For example, expression of *ERECTA* protein in the plant can be modified by ectopic expression of an *ERECTA* allele in the
10 plant, or alternatively, by reducing endogenous *ERECTA* expression using an inhibitory RNA (eg, antisense or RNAi).

A fifth aspect of the present invention provides a plant having enhanced transpiration efficiency, wherein said plant is produced by a method described herein.

15

- Plants that have enhanced transpiration efficiency show increased levels of growth under normal growth conditions, thereby increasing their biomass. Accordingly, a further aspect of the present invention provides a method of increasing the biomass of a plant comprising enhancing the level of expression of an *ERECTA* gene or allelic
20 variant thereof or protein coding region thereof in said plant.

In one embodiment, the method further includes the step of selecting a plant that has an increased biomass when compared to an unmodified plant. Methods of determining the biomass of a plant are well known to those skilled in the art and/or described herein.

25

In one embodiment, the level of expression is enhanced by genetic modification of a control sequence, for example a promoter sequence, associated with the *ERECTA* gene or allelic variant thereof.

- 30 In another embodiment, the level of expression is enhanced by introducing (eg., by classical breeding, introgression or recombinant means) an *ERECTA* gene or allelic

variant thereof or the protein encoding region thereof to a plant. Preferably, the *ERECTA* gene or allelic variant or protein-encoding region comprises a nucleotide sequence selected from the group consisting of:

- a sequence having at least about 55% identity to a sequence selected from the group
- 5 consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43 and SEQ ID NO: 44; and
- 10 a sequence encoding an amino acid sequence having at least about 55% identity to an amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 20 and SEQ ID NO: 45.

The plant into which the gene etc is introduced is preferably selected from the group consisting of *Arabidopsis thaliana*, rice, sorghum, wheat and maize.

20

A further aspect of the present invention provides a method of increasing the resistance of a plant to an environmental stress comprising enhancing the level of expression of an *ERECTA* gene or allelic variant thereof or protein coding region thereof in said plant.

- 25 As used herein the term "environmental stress" shall be taken in its broadest context to mean one or more environmental conditions that reduce the ability of a plant to grow, survive and/or produce seed/grain. In one embodiment, an environmental stress that affects the ability for a plant to grow, survive and/or produce seed/grain is a condition selected from the group consisting of increased or decreased CO₂ levels, increased or
- 30 decreased temperature, increased or decreased rainfall, increased or decreased

humidity, increased salt levels in the soil, increased soil strength and compaction and drought.

5 In one embodiment, the method further includes the step of selecting a plant that has an altered resistance to an environmental stress when compared to an unmodified plant is selected. Methods of determining the resistance of a plant to environmental stress are well known to those skilled in the art and/or described herein.

10 In one embodiment, the level of expression is enhanced by genetic modification of a control sequence, for example a promoter sequence, associated with the *ERECTA* gene or allelic variant thereof.

In another embodiment, the level of expression is enhanced by introducing (eg., by classical breeding, introgression or recombinant means) an *ERECTA* gene or allelic
15 variant thereof or the protein encoding region thereof to a plant. Preferably, the *ERECTA* gene or allelic variant or protein-encoding region comprises a nucleotide sequence selected from the group consisting of:

a sequence having at least about 55% identity to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID
20 NO: 9, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID
25 NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43 and SEQ ID NO: 44; and

a sequence encoding an amino acid sequence having at least about 55% identity to an amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO:
30 20 and SEQ ID NO: 45.

The plant into which the gene etc is introduced is preferably selected from the group consisting of *Arabidopsis thaliana*, rice, sorghum, wheat and maize.

A further aspect of the present invention provides a plant having increased resistance to environmental stress, wherein said plant is produced by a method described herein.

Both temperature and available moisture have been shown to dramatically influence pollination and grain/seed development, processes known as seed-set and grain-filling. Accordingly, a method that produces a plant that is resistant to environmental stress, ie a plant that has increased transpiration efficiency, results in increased or more efficient grain-filling and greater seed number. As *ERECTA* is expressed during flowering or pod development this gene or an allelic variant thereof is useful for increasing grain-filling in a plant.

Accordingly, a further aspect of the present invention provides a method of increasing seed or grain weight in a plant comprising enhancing the level of expression of an *ERECTA* gene or allelic variant thereof or protein coding region thereof in said plant.

In one embodiment, the method further includes the step of selecting a plant that has increased seed or grain weight when compared to an unmodified plant is selected. Methods of determining seed or grain weight are well known to those skilled in the art and/or described herein.

In one embodiment, the level of expression is enhanced by genetic modification of a control sequence, for example a promoter sequence, associated with the *ERECTA* gene or allelic variant thereof.

In another embodiment, the level of expression is enhanced by introducing (eg., by classical breeding, introgression or recombinant means) an *ERECTA* gene or allelic variant thereof or the protein encoding region thereof to a plant. Preferably, the

ERECTA gene or allelic variant or protein-encoding region comprises a nucleotide sequence selected from the group consisting of:

- a sequence having at least about 55% identity to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43 and SEQ ID NO: 44; and
- a sequence encoding an amino acid sequence having at least about 55% identity to an amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 20 and SEQ ID NO: 45.

The plant into which the gene etc is introduced is preferably selected from the group consisting of *Arabidopsis thaliana*, rice, sorghum, wheat and maize.

- 20 A further aspect of the present invention provides a plant having increased seed or grain weight, wherein said plant is produced by a method described herein.

- A still further aspect of the present invention provides a method of modulating the number of seeds produced by a plant comprising enhancing the level of expression of
- 25 an *ERECTA* gene or allelic variant thereof in said plant.

- In one embodiment, the method further includes the step of selecting a plant that has an increased number of seeds when compared to an unmodified plant is selected. Methods of determining seed or grain number are well known to those skilled in the art and/or
- 30 described herein.

In one embodiment, the level of expression is enhanced by genetic modification of a control sequence, for example a promoter sequence, associated with the *ERECTA* gene or allelic variant thereof.

- 5 In another embodiment, the level of expression is enhanced by introducing (eg., by classical breeding, introgression or recombinant means) an *ERECTA* gene or allelic variant thereof or the protein encoding region thereof to a plant. Preferably, the *ERECTA* gene or allelic variant or protein-encoding region comprises a nucleotide sequence selected from the group consisting of:
- 10 a sequence having at least about 55% identity to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID
- 15 NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43 and SEQ ID NO: 44; and
- 20 a sequence encoding an amino acid sequence having at least about 55% identity to an amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 20 and SEQ ID NO: 45.

The plant into which the gene etc is introduced is preferably selected from the group consisting of *Arabidopsis thaliana*, rice, sorghum, wheat and maize.

A further aspect of the present invention provides a plant having an increased number of seeds, wherein said plant is produced by a method described herein.

30 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a is a graphical representation showing the CO₂ assimilation rates ($\mu\text{mol C m}^{-2} \text{ s}^{-1}$) of several genotypes of *A. thaliana*. Measurements were completed on rosette leaves during bolting and flowering stages. Plants were grown on fertilised soil. The genotypes of plants are indicated on the x-axis, and CO₂ assimilation rates indicated on the ordinate. Col indicates a genetic background of the ecotype Columbia. Ld indicates a genetic background of the ecotype Landsberg. Plants expressing wild type *ERECTA* alleles were either in a Col (Col4-*ER*) or Ld (Ld-*ER*) background. Plants that were homozygous for a mutant *er* allele were either in a Ld background (Ld-*er1*) or in a Col background (Col-*er105* or Col-*er2* (line 3401 at NASC, also named Col-*er106* by Torii and collaborators (see Lease et al. 2001, *New Phytologist*, 151:133-143)). Plants designated as F1 (Col-*ER* x Ld-*er*) were heterozygous *ER/er1*. Data indicate that, in a Col background, the *er105* mutation leads to reduced CO₂ assimilation rate, whilst the *er1* mutation enhances CO₂ assimilation rate in a Ld background.

Figure 1b is a graphical representation showing the stomatal conductance ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) of several genotypes of *A. thaliana* (same plants as Fig.1a). The genotypes of plants are indicated on the x-axis and are the same as described in the legend to Figure 1a. Stomatal conductances are indicated on the ordinate. Data indicate that, in a Col background, the *er2/er106* mutation significantly enhances stomatal conductance, whilst the *er1* mutation significantly enhances stomatal conductance in a Ld background.

Figure 1c is a graphical representation showing the transpiration efficiency of ($\text{mmol C mol H}_2\text{O}^{-1}$) of several genotypes of *A. thaliana*, as determined by the ratio of CO₂ assimilation rate to stomatal conductance. The genotypes of plants are indicated on the x-axis and are the same as described in the legend to Figure 1a. Transpiration efficiency is indicated on the ordinate. Data indicate that transpiration efficiency is enhanced in plants expressing a wild type *ER* allele relative to a mutant *er* allele, in both Ld and Col backgrounds. The lowest transpiration efficiency was observed for plants that are homozygous for the *er105* allele (ie. Col-*er105*), consistent with the fact that this allele inhibits *ERECTA* expression. From the data in Figures 1a-1c, it is apparent that the

lower transpiration efficiency of plants expressing the *er105* allele is largely due to a reduced CO₂ fixation rate, whereas for both the *er2/er106* and *er1* alleles, reduced transpiration efficiency is largely due to enhanced stomatal conductance. The transpiration efficiency of the F1 heterozygote plant was intermediate between the
5 transpiration efficiencies of its parents, suggesting codominance of these alleles. The F1, however, had a transpiration efficiency closer to that of the pollen donor parent, Ld-*er1*.

Figure 2a is a graphical representation showing the stomatal densities (Number of
10 stomata mm⁻² leaf) for several genotypes of *A. thaliana* in three independent experiments. The genetic backgrounds of plants are indicated on the x-axis (Col, Columbia; Ld, Landsberg), and stomatal densities are indicated on the ordinate. Plant genotypes are indicated at the top of each bar, as follows: plants expressing wild type *ERECTA* alleles in a Col background were Col4*ER* or Col1*ER* (hatched bars); plants
15 expressing wild type *ERECTA* alleles in a Ld background were *ER* (open bars); plants expressing mutant *erecta* alleles in a Col background were either *er105* or *er2/106* (Col filled boxes); and plants expressing the mutant *er1* allele in a Ld background were *er1* (Ld filled boxes). Columns designated a,b are data from two experiments where plants were grown in soil in the absence of fertiliser. The set of columns at the right of the
20 figure are from a third experiment where the same plants were grown in soil comprising fertiliser. Data indicate that, in a Col background, the *er105* mutation and *er2/106* mutation enhances stomatal density, which in part accounts for the enhanced stomatal conductances and reduced transpiration efficiencies of plants expressing these alleles (Figures 1b and 1c). The general effect of these alleles is not dependent on the
25 nutrient status of the soil. In contrast, the *er1* allele only enhanced stomatal density of Ld plants when fertiliser was absent, suggesting that in this ecotype enhanced stomatal aperture accounted for the enhanced stomatal conductances and reduced transpiration efficiencies measured in the *er1* mutant under ample nutrient supply (Figures 1b, 1c). The *er1* mutation therefore affects both stomatal aperture and stomatal density but the
30 relative contributions of these effects to enhanced stomatal conductance per unit leaf

area depend on environmental factors and plant nutrient status, and on genetic background.

Figure 2b is a graphical representation showing the epidermal cell size (surface area, μm^2) for several genotypes of *A. thaliana* in three independent experiments. The genetic backgrounds and genotypes of plants are indicated on the x-axis and at the tops of each column, respectively, as in the legend to Figure 2a. The ordinate indicates epidermal cell size. Columns designated a,b are data from two experiments where plants were grown in soil in the absence of fertiliser. The set of columns at the right of the figure are from a third experiment where the same plants were grown in soil comprising fertiliser. Data indicate that, in a Col background, the *er105* mutation and *er2/er106* mutation significantly reduce epidermal cell size ie increase the number of epidermal cells per unit leaf area. This reveals that the ER gene has effects on leaf histogenesis which, beyond their consequences on stomatal densities, may also directly affect leaf capacity for photosynthesis and therefore transpiration efficiency, (Figures 1b and 1c). The general effects of these alleles are not dependent on the nutrient status of the soil. In contrast, in a Ld background, the *er1* allele reduced epidermal cell size only when fertiliser was absent.

Figure 2c is a graphical representation showing the stomatal index for several genotypes of *A. thaliana* in three independent experiments. The genetic backgrounds and genotypes of plants are indicated on the x-axis and at the tops of each column, respectively, as in the legend to Figure 2a. The ordinate indicates stomatal index, as determined from the ratio of stomatal density to epidermal cell density. Columns designated a,b are data from two experiments where plants were grown in soil in the absence of fertiliser. The set of columns at the right of the figure are from a third experiment where the same plants were grown in soil comprising fertiliser. Data indicate that the *er* mutations tested do not significantly modify stomatal index in Col background (because increases in stomatal density are correlated to increases in epidermal cell numbers in the Col mutant plants) but does so in Landsberg background. Accordingly, the *ER* gene does appear to directly modify stomatal development *per se*.

Taken together Figures 2a-c therefore show that the *ERECTA* gene has two types of effects on leaf stomatal conductance: a) developmental, b) biophysical and/or biochemical. The expression of these effects and impact on transpiration rate vary with genetic background, suggesting interactions with other genes that are polymorphic
5 between the Col and Ld ecotypes, and also with nutrient status.

Figure 3 is a graphical representation showing carbon isotope composition (y-axis; in per mil, for vegetative rosettes) for 7 different experimental runs (numbers 1-7) carried out under growth cabinet conditions and glasshouse conditions. For each run, the left-
10 hand side bar shows the mean value of carbon isotope composition for lines carrying the *ERECTA* allele, while the right-hand side bar shows the mean value across lines with the *erecta* allele. In all cases, $\delta^{13}\text{C}$ isotopic composition values for the er-lines are more negative than those for ER lines, indicative of lower transpiration efficiencies.

15 Figure 4a is a graphical representation showing *ERECTA* gene copy number and expression levels in transgenic T2 *A. thaliana* plants homozygous for an ER transgene. These lines were generated by transforming the Col-er2/106 mutant with the wild type ER gene under the 35S promoter. Effective transformation was ascertained and *ERECTA* expression levels were quantified in several independent transformants using
20 real-time quantitative PCR (ABI PRISM 7700, Sequence Detection System User Bulletin #2, 1997). Copy number (y-axis) is indicated as a function of the plant line, following normalisation of *ERECTA* relative to the copy number of a control gene (18S ribosomal RNA gene). The expression of the 18S rRNA gene was shown independently not to be affected by changes in ER expression. Line 143 is null control
25 (no insert). Lines 145, 165, 169 and 279 are transformed lines carrying the *ERECTA* allele. All ER transgenic lines, except line 145, show increased mRNA copy number: from 4 to 9.5 fold increase compared with the null control.

Figure 4b is a graphical representation showing *ERECTA* gene copy number and
30 expression levels in transgenic T2 *A. thaliana* plants homozygous for an ER transgene, and generated by transformation of the Col-er105 mutant. Effective transformation

was ascertained and *ERECTA* expression levels were quantified in several independent transformants using real-time quantitative PCR (ABI PRISM 7700, Sequence Detection System User Bulletin #2. 1997). Copy number (y-axis) is indicated as a function of the plant line, following normalisation of *ERECTA* relative to the copy number of a control gene (18S ribosomal RNA gene). The expression of the 18S rRNA gene was shown independently not to be affected by changes in ER expression. Line 18 is a null control line (no ER insert, ie similar to Col-er105). Lines 8, 19, 29 and 61 are transgenic lines carrying the *ERECTA* allele. All *ER* transgenic lines show increased mRNA copy number: from 10 to 170 fold increase compared with the null control.

10

Figure 4c is a graphical representation showing *ERECTA* gene copy number and expression levels in Col and Ld ER ecotypes and in one Ld-ER transgenic line (3-7K) generated by transformation of the Ld-er1 ecotype (NW20) with the ER wild type gene under control of the 35S promoter. Effective transformation was ascertained and *ERECTA* expression levels were quantified in several independent transformants using real-time quantitative PCR (ABI PRISM 7700, Sequence Detection System User Bulletin #2. 1997). Copy number (y-axis) is indicated as a function of the plant line, following normalisation of *ERECTA* relative to the copy number of a control gene (18S ribosomal RNA gene). The expression of the 18S rRNA gene was shown independently not to be affected by changes in ER expression. Lines 933, 1093 and 3176 are the non-transformed Columbia-*ERECTA* ecotypes Col-4, Col-0 and Col-1. Line 105c is a Col-er105 line (knockout for ER), used for generating transgenic lines shown in Figure 4b. Lines labelled 2c and 3401 on the X-axis describe Col-er2/106 (2 batches of seeds, used for generating transgenic lines shown in Figure 4a). Line NW20 is Ld-er1. Line 3-7K is a Ld-ER transformant, obtained from transformation of Ld-er1 with the *ERECTA* allele. Line 3177 is the Ld-ER ecotype, near-isogenic to NW20.

Figure 5a is a graphical representation of a first experiment showing copy number of the mRNA transcription product of the rice *ERECTA* gene in various plant organs/parts, cv Nipponbare. L= mature leaf blades; YL= young expanding leaves, still enclosed in sheaths of older leaves; R= mature root; YR= young root; SH= sheaths; INF= unfolded

30

young panicle still enclosed in sheaths; O7: young panicles. Rice *ERECTA* mRNA copy numbers were determined by quantitative real-time PCR, with 18S mRNA as internal control gene for normalization of results. The values on the y-axis describe fold increases of rice *ERECTA* mRNA in various parts compared to the L sample (mature leaves) set to a value of 1 for normalization. Data show a similar expression pattern as the *ERECTA* gene in Arabidopsis (see Torii et al. 1996) ie preferential expression in young meristematic tissues, especially in reproductive organs.

Figure 5b is a graphical representation of a second experiment showing copy number of the mRNA transcription product of the rice *ERECTA* gene in various plant organs/parts. L= mature leaf blades; YL= young expanding leaves, still enclosed in sheaths of older leaves; R= mature root; YR= young root; SH= sheaths; INF= unfolded young panicle still enclosed in sheaths; O7: young panicles. Rice *ERECTA* mRNA copy numbers were determined by quantitative real-time PCR, with 18S mRNA as internal control gene for normalization of results. The values on the y-axis describe fold increases of rice *ERECTA* mRNA in various parts compared to the L sample (mature leaves) set to a value of 1 for normalization. Data confirm those shown in Figure 5a.

Figure 6 is a graphical representation showing leaf transpiration efficiency ($\text{mmol C mol H}_2\text{O}^{-1}$, Figure 6a), calculated from the direct measurements of leaf CO_2 assimilation rate ($\mu\text{mol C m}^{-2} \text{s}^{-1}$, Figure 6b) and stomatal conductance ($\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$, Figure 6c) by gas exchange techniques, under 350 ppm CO_2 (ie same as ambient $[\text{CO}_2]$ during seedling growth; left hand bar in each pair of bars) and 500ppm CO_2 (right hand bar in each pair of bars), for Ld-er1, and two Ld_ER lines: line T2(+ER), a T2 transgenic line homozygous for an ER transgene in the Ld-er1 background and line 3177, an ER ecotype near-isogenic to Ld-er1 (NASC Stock Centre information). Genotypes are shown at the bottom of the figure. Leaf temperature during measurements was controlled at 22°C, leaf to air vapour pressure deficit at around 8mb.

Figure 7 is a graphical representation showing leaf transpiration efficiency ($\text{mmol C mol H}_2\text{O}^{-1}$, Figure 7a), calculated from the direct measurements of leaf CO_2

assimilation rate ($\mu\text{mol C m}^{-2} \text{ s}^{-1}$, Figure 7b) and stomatal conductance ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$, Figure 7c) by gas exchange techniques, under 350 ppm CO_2 (ie same as ambient $[\text{CO}_2]$ during seedling growth; left hand bar in each pair of bars) and 500ppm CO_2 (right hand bar in each pair of bars), for 4 genotypes: Col4 (ER) (left hand pair), Ld (er1) (right hand pair) and their F_1 progeny (middle two pairs). Genotypes are shown at the bottom of the figure.

Figure 8 is a graphical representation showing stomatal conductance and epidermal anatomy at 350ppm CO_2 in the genotypes described in Figures 6 and 7 and shown at the bottom of the figure. The insertion of ER transgene (line T2+ER) caused a decreased in stomatal conductance compared to the Ld-er1 line (Figure 8a), which was in part due to a decrease in stomatal density (see Figure 8c). These two effects again indicate complementation. Together Figure 8b and 8c show that the decrease in stomatal density is relatively more important than that in epidermal cell density, indicating an effect of the transgene on epidermis development.

Figure 9 is a graphical representation showing a comparison of stomatal density and epidermal cell area in a range of Col_er lines carrying mutations in the ER gene (bars 1 to 8 Fig. 9a; bar 1 to 7 in Fig. 9b, mutants er105, er106, 108, 111, 114, 116, 117, as described in Lease et al. 2001; a gift from Dr Keiko Torii) and in Col-ER wild type ecotypes (bars 9-11 or 8-10 in Figures 9a and 9b, respectively: Col0, background ecotype for these mutants; Col1, Col4 (ColER parental line for QTL analysis of Lister and Dean's RILs), two Ld_er1 lines (NW20 and CS20, bars 12&13 and 11&12 in Figs 9a and 9b respectively, two very similar lines according to NASC; NW20 is the other parental line for Lister and Dean's RILs) and finally line T2+ER, a transgenic Ld-ER line carrying the ER wild type gene in Ld-er1 background (extreme right hand bar on the figure).

Figure 10 is a graphical representation showing carbon isotopic composition (per mil, y-axis) in a range of lines (numbered 1 to 19 on the x-axis): Col-er mutants (line 1-14); the Col0 background ecotype (line 15); Ld-er1 lines (lines 16 and 17); an Ld-ER near

isogenic ecotype to Ld-er1 (line 18, line 3177 at NASC), and a transgenic T2 Ld-ER line (line numbered 19) obtained by transformation of Ld-er1 mutant with a construct carrying the wild type ER allele. The data show that the ER allele gives less negative values indicative of increased transpiration efficiency.

5

Figure 11 is a graphical representation showing direct measurements of transpiration efficiency in Col-er mutants transformed with ER transgene, under both high and low air humidity, such as occurs during hot temperature events causing or associated with drought. Transpiration efficiency was measured by gas exchange techniques on mature
10 leaves of vegetative Arabidopsis rosettes, as a function of leaf-to-air vapour pressure difference (vpd) ie air humidity around the leaves. The higher the vpd, the drier the air. Solid circles describe measurements for 5 independent transgenic T2 lines homozygous for an ER transgene; these lines were generated by transforming the Col-er105 mutant (empty squares) with a construct carrying the ER allele under control of the 35S
15 promoter. Data for null lines (ie lines that went through transgenesis but do not carry the ER transgene) are represented by solid squares. This figure demonstrates complementation, across the whole range of humidity tested, with the transpiration efficiencies in T2 ER lines being greater than those in the complemented Col-er105 mutant, and similar to those measured in the Col0-ER ecotype (empty triangles;
20 background ecotype for Col-er105).

Figure 12 is a graphical representation of an alignment of isolated sequences with the entire coding region of the wheat ortholog of ERECTA. The position of each of the isolated sequences is shown relative to the wheat ortholog of ERECTA. Sequences are
25 represented by either SEQ ID NO. or gene accession number.

Figure 13 is a graphical representation of an alignment of isolated sequences with the entire coding region of the maize ortholog of ERECTA. The position of each of the isolated sequences is shown relative to the maize ortholog of ERECTA. Sequences are
30 represented by either SEQ ID NO. or gene accession number.

Figure 14 is a graphical representation of a pairwise sequence alignment of the ERECTA proteins isolated from Arabidopsis (SEQ ID NO: 2), maize (SEQ ID NO: 45), rice (SEQ ID NO: 3), Sorghum (SEQ ID NO: 5) and wheat (SEQ ID NO: 20). The alignment was performed using CLUSTALW multiple sequence alignment tool.

- 5 Residues that are conserved between all species are indicated by asterisks (*). Conservation of the groups STA NEQK NHQK NDEQ QHRK MILV MILF HY or FYW is indicated by “:”. Conservation of the groups CSA ATV SAG STNK STPA SGND SNDEQK NDEQHK NEQHRK FVLIM HFY is indicated by “.”. Gaps are indicated by dashes “-”.

10

Figure 15 is a graphical representation of a phylogenetic tree indicating the relationship between each of the ERECTA proteins isolated from Arabidopsis (SEQ ID NO: 2), maize (SEQ ID NO: 45), rice (SEQ ID NO: 3), Sorghum (SEQ ID NO: 5) and wheat (SEQ ID NO: 20).

15

DETAILED DESCRIPTION OF THE INVENTION

Loci for transpiration efficiency and their identification

- One aspect of the invention provides a locus associated with the genetic variation in transpiration efficiency of a plant, wherein said locus comprises a nucleotide sequence linked genetically to an *ERECTA* locus in the genome of the plant.
- 20

- As used herein, the term “locus” shall be taken to mean the location of one or more genes in the genome of a plant that affects a quantitative characteristic of the plant, in particular the transpiration efficiency of a plant. In the present context, a “quantitative characteristic” is a phenotype of the plant for which the phenotypic variation among different genotypes is continuous and cannot be separated into discrete classes, irrespective of the number of genes that determine or control the phenotype, or the magnitude of genetic effects that single gene has in determining the phenotype, or the magnitude of genetic effects of interacting genes.
- 25
- 30

By "associated with the genetic variation in transpiration efficiency of a plant" means that a locus comprises one or more genes that are expressed to determine or regulate the transpiration efficiency of a plant, irrespective of the actual rate of transpiration achieved by the plant under a specified environmental condition.

5

Preferably, the locus of the invention is linked to or comprises an *ERECTA* allele or *erecta* allele, or a protein-encoding portion thereof

As used herein, the term "*ERECTA*" shall be taken to refer to a wild type allele
10 comprising the following domains GTIGYIDPEYARTS, GAAQGLAYLHHDC, and
TENLSEKXIIGYGASSTVYKC domains, wherein X means Y or H, or domains more
than 94 % identical to these domains. To the inventors' knowledge no other protein
comprises these domains. Preferred *ERECTA* alleles comprise a nucleotide sequence
having at least about 55% overall sequence identity to the protein-encoding region of
15 any one of the exemplified *ERECTA* alleles described herein, particularly any one of
SEQ ID Nos: 1, 3, 5, 7, 9, 11, 13, or 15. Preferably, the percentage identity to any one
of said SEQ ID NOs: is at least about 59-61%, or 70% or 80%, and more preferably at
least about 90%, and still more preferably at least about 95% or 99%.

20 Preferred *ERECTA* alleles are derived from, or present in, the genome of a plant that is
desiccation or drought intolerant, or poorly adapted for growth in dry or arid
environments, or that suffers from reduced vigor or growth during periods of reduced
rainfall or drought, or from the genome of a plant with increased growth rate or growth
duration or partitioning of C to shoot and harvested parts under well-watered
25 conditions.

More preferably, an *ERECTA* allele is derived from, or present in, the genome of a
brassica plant, broad acre crop plant, perennial grass (eg. of the subfamily Pooidae, or
the Tribe Poeae), or tree. Even more preferably, an *ERECTA* allele is present in or
30 derived from the genome of a plant selected from the group consisting of barley, wheat,

rye, sorghum, rice, maize, *Phalaris aquatica*, *Dactylus glomerata*, *Lolium perenne*, *Festuca arundinacea*, cotton, tomato, soybean, oilseed rape, poplar, and pine.

The term “*erecta*” shall be taken to mean any allelic variant of the wild-type *ERECTA* allele that modifies transpiration efficiency of a plant.

Preferred *erecta* alleles include the following *A. thaliana erecta* alleles derived from Columbia (Col) and Landsberg *erecta* (er) lines.

10

Erecta alleles ¹	Genomic position	Lesion	Affected domain
Ler er-1	2249	T→A	PK
Col er-101	6565	T→A	PK
Col er-102/106	6565	T→A	PK
Col er-103	846	G→A	LRR10
Col er-105	foreign DNA insert between +5 and +1056	insertion	Null allele
Col er-108	5649	G→A	
Col er-111	5749	G→A	Untranslated region between LRR and transmembrane domains
Col er-113	3274	C→T	
Col er-114	6807	G→A	PK
Col er-115	3796	C→T	
Col er-116	6974	G→A	PK
Col er-117	5203	G→A	LRR18

¹ alleles described by Lease et al. 2001, New Phytologist, 151: 133-143, except for Ler er-1, Col er-103 and Col-er105 which were described in Torii et al., 1996, The Plant Cell 8:735-746

15

The present invention clearly encompasses an *erecta* allele derived from, or present in, the genome of a plant that is desiccation or drought intolerant, or poorly adapted for growth in dry or arid environments, or that suffers from reduced vigor or growth during periods of reduced rainfall or drought, or from the genome of a plant with increased

20

growth rate or growth duration or partitioning of C to shoot and harvested parts under well-watered conditions.

More preferably, an *erecta* allele is derived from, or present in, the genome of a
5 brassica plant, broad acre crop plant, perennial grass (eg. of the subfamily Pooidae, or
the Tribe Poeae), or tree. Even more preferably, an *erecta* allele is present in or derived
from the genome of a plant selected from the group consisting of barley, wheat, rye,
sorghum, rice, maize, *Phalaris aquatica*, *Dactylus glomerata*, *Lolium perenne*, *Festuca*
arundinacea, cotton, tomato, soybean, oilseed rape, poplar, and pine.

10

For the purposes of nomenclature, the nucleotide sequence of the *Arabidopsis thaliana*
ERECTA protein-encoding region and the 5'-untranslated region (UTR) and 3'-UTR, is
provided herein as SEQ ID NO: 1. The amino acid sequence of the polypeptide
encoded by SEQ ID NO: 1 is set forth herein as SEQ ID NO: 2.

15

A particularly preferred *ERECTA* allele from rice (*Oryza sativa*) is derived from
chromosome 6 of that plant species. For the purposes of nomenclature, the protein-
encoding region of the rice *ERECTA* gene is provided herein as SEQ ID NO: 3. The
amino acid sequence of the polypeptide encoded by SEQ ID NO: 3 is set forth herein as
20 SEQ ID NO: 4.

25

A particularly preferred *ERECTA* gene derived from the genome of *Sorghum bicolor*, is
provided herein as SEQ ID NO: 5. The amino acid sequence of the polypeptide
encoded by SEQ ID NO: 5 is set forth herein as SEQ ID NO: 6.

A further exemplary *ERECTA* gene derived from *A. thaliana* is provided herein as SEQ
ID NO: 7. The amino acid sequence of the polypeptide encoded by SEQ ID NO: 7 is
set forth herein as SEQ ID NO: 8.

A further exemplary *ERECTA* gene derived from *A. thaliana* is provided herein as SEQ ID NO: 9. The amino acid sequence of the polypeptide encoded by SEQ ID NO: 9 is set forth herein as SEQ ID NO: 10.

- 5 Fragments of an exemplary *ERECTA* gene derived from the genome of wheat are provided herein as SEQ ID NOs: 11 to 18.

An exemplary *ERECTA* gene derived from the genome of wheat is provided herein as SEQ ID NO: 19. The amino acid sequence of the polypeptide encoded by SEQ ID NO:
10 19 is set forth herein as SEQ ID NO: 20.

Fragments of an exemplary *ERECTA* gene derived from the genome of maize are provided herein as SEQ ID NOs: 21 to 43.

- 15 An exemplary *ERECTA* gene derived from the genome of maize is provided herein as SEQ ID NO: 44. The amino acid sequence of the polypeptide encoded by SEQ ID NO: 44 is set forth herein as SEQ ID NO: 44.

The present invention clearly contemplates the presence of multiple genes that are
20 genetically linked or map to the specified *ERECTA* locus on chromosome 2. Without being bound by any theory or mode of action, such multiple linked genes may interact, such as, for example, by epistatic interaction, to determine the transpiration efficiency phenotype.

- 25 The present invention also contemplates the presence of different alleles of any gene that is linked to the *ERECTA* locus, wherein said allele is expressed to determine the transpiration efficiency phenotype. In one embodiment, such alleles are identified by detecting a particular transpiration efficiency phenotype that is linked to the expression of the particular allele. Alternatively, or in addition, the different alleles linked to a
30 locus are identified by detecting a structural polymorphism in DNA (eg. a restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism

(AFLP), single strand chain polymorphism (SSCP), and the like), that is linked to a particular transpiration efficiency phenotype.

The present invention clearly encompasses all interacting genes and/or alleles that are
5 genetically linked to an *ERECTA* locus and are expressed to determine a transpiration efficiency phenotype. Such linked interacting genes and/or alleles will map to an *ERECTA* locus and be associated with the transpiration efficiency of that plant. Preferably, such interacting genes and/or alleles comprise a protein-encoding portion of a gene positioned within the *ERECTA* locus of the genome that is associated with the
10 transpiration efficiency of that plant.

Homologs and/or orthologs of the exemplified alleles are clearly encompassed by the invention. Those skilled in the art are aware that the terms "homolog" and "ortholog" refer to functional equivalent units. In the present context, a homolog or ortholog of a
15 gene that maps to an *ERECTA* locus shall be taken to mean any gene from a plant species that is functionally equivalent to a gene that maps to an exemplified *ERECTA* locus, and comprises a protein-encoding region in its native plant genome that shares a degree of structural identity or similarity with a protein-encoding region of the exemplified *ERECTA* gene.

20

Preferably, a homologous or orthologous gene from a plant other than *A. thaliana* will be associated with the transpiration efficiency of said plant and be linked to a protein-encoding region in its native plant genome that comprises a nucleotide sequence having at least about 55% overall sequence identity to a protein-encoding region linked to the
25 *ERECTA* locus. Even more preferably, the percentage identity will be at least about 59-61% or 70% or 80%, still more preferably at least about 90%, and even still more preferably at least about 95%.

In determining whether or not two nucleotide sequences fall within a particular
30 percentage identity limitation recited herein, those skilled in the art will be aware that it is necessary to conduct a side-by-side comparison or multiple alignment of sequences.

In such comparisons or alignments, differences may arise in the positioning of non-identical residues, depending upon the algorithm used to perform the alignment. In the present context, reference to a percentage identity between two or more nucleotide sequences shall be taken to refer to the number of identical residues between said
5 sequences as determined using any standard algorithm known to those skilled in the art. For example, nucleotide sequences may be aligned and their identity calculated using the BESTFIT program or other appropriate program of the Computer Genetics Group, Inc., University Research Park, Madison, Wisconsin, United States of America (Devereaux *et al*, *Nucl. Acids Res.* 12, 387-395, 1984). In determining percentage
10 identity of nucleotide sequences using a program known in the art or described herein, it is preferable that default parameters are used.

Alternatively, or in addition, a homologous or orthologous *ERECTA* or *erecta* allele will be associated with the transpiration efficiency of a plant and be linked to a protein-
15 encoding region in its native plant genome that comprises a nucleotide sequence that encodes a polypeptide having at least about 55% overall sequence identity to a polypeptide encoded by a protein-encoding region linked to the *ERECTA* locus. Preferably, the percentage identity at the amino acid level will be at least about 59-61% or 70% or 80%, more preferably at least about 90%, and still more preferably at least
20 about 95%.

In determining whether or not two amino acid sequences fall within these percentage limits, those skilled in the art will be aware that it is necessary to conduct a side-by-side comparison or multiple alignment of sequences. In such comparisons or alignments,
25 differences will arise in the positioning of non-identical residues, depending upon the algorithm used to perform the alignment. In the present context, reference to a percentage identity or similarity between two or more amino acid sequences shall be taken to refer to the number of identical and similar residues respectively, between said sequences as determined using any standard algorithm known to those skilled in the art.
30 For example, amino acid sequence identities or similarities may be calculated using the GAP program and/or aligned using the PILEUP program of the Computer Genetics

Group, Inc., University Research Park, Madison, Wisconsin, United States of America (Devereaux *et al.*, 1984, *supra*). The GAP program utilizes the algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48, 443-453, 1970, to maximize the number of identical/similar residues and to minimize the number and length of sequence gaps in the alignment. Alternatively or in addition, wherein more than two amino acid sequences are being compared, the ClustalW program of Thompson *et al.*, *Nucl. Acids Res.* 22, 4673-4680, 1994, is used. In determining percentage identity of amino acid sequences using a program known in the art or described herein, it is preferable that default parameters are used.

10

Alternatively, or in addition, a homologous or orthologous *ERECTA* or *erecta* allele will be associated with the transpiration efficiency of a plant and be linked to a protein-encoding region in its native plant genome that hybridizes to nucleic acid that comprises a sequence complementary to a protein-encoding region linked to an *ERECTA* locus, such as, for example, from *A. thaliana*, rice, sorghum, maize, wheat or rice. Preferably, such homologs or orthologs will be identified by hybridization under at least low stringency conditions, and more preferably under at least moderate stringency or high stringency hybridization conditions.

20 For the purposes of defining the level of stringency, a low stringency is defined herein as being a hybridization or a wash carried out in 6xSSC buffer, 0.1% (w/v) SDS at 28°C or alternatively, as exemplified herein. Generally, the stringency is increased by reducing the concentration of salt in the hybridization or wash buffer, such as, for example, by reducing the concentration of SSC. Alternatively, or in addition, the stringency is increased, by increasing the concentration of detergent (eg. SDS). Alternatively, or in addition, the stringency is increased, by increasing the temperature of the hybridization or wash. For example, a moderate stringency can be performed using 0.2xSSC to 2xSSC buffer, 0.1% (w/v) SDS, at a temperature of about 42°C to about 65°C. Similarly, a high stringency can be performed using 0.1xSSC to 0.2xSSC buffer, 0.1% (w/v) SDS, at a temperature of at least 55°C. Conditions for performing nucleic acid hybridization reactions, and subsequent membrane washing, are well

understood by one normally skilled in the art. For the purposes of further clarification only, reference to the parameters affecting hybridization between nucleic acid molecules is found in Ausubel *et al.*, *In: Current Protocols in Molecular Biology*, Greene/Wiley, New York USA, 1992, which is herein incorporated by reference.

5

A number of mapping methods for determining useful loci and estimating their effects have been described (eg. Edwards *et al.*, *Genetics* 116, 113-125, 1987; Haley and Knott, *Heredity* 69, 315-324, 1992; Jiang and Zeng, *Genetics* 140, 1111-1127, 1995; Lander and Botstein, *Genetics* 121, 185-199, 1989; Jansen and Stam, *Genetics* 136,
10 1447-1455, 1994; Utz and Melchinger, *In: Biometrics in Plant Breeding: Applications of Molecular Markers*. Proc. Ninth Meeting of the EUCARPIA Section Biometrics in Plant Breeding, 6 - 8 July 1994, Wageningen, The Netherlands, (J.W. van Ooijen and J. Jansen, eds), pp195-204, 1994; Zeng, *Genetics* 136, 1457-1468, 1994). In the present context, these methods are applied to identify the major component(s) of the total
15 genetic variance that contribute(s) to the variation in transpiration efficiency of a plant, such as, for example, determined by the measurement of carbon isotope discrimination (Δ). More particularly, the segregation of known markers is used to map and/or characterize an underlying locus associated with transpiration efficiency. The locus method involves searching for associations between the segregating molecular markers
20 and transpiration efficiency in a segregating population of plants, to identify the linkage of the marker to the locus.

To discover a marker/locus linkage, a segregating population is required. Experimental populations, such as, for example, an F2 generation, a backcross (BC) population,
25 recombinant inbred lines (RIL), or double haploid line (DHL), can be used as a mapping population. Bulk segregant analysis, for the rapid detection of markers at specific genomic regions using segregating populations, is described by Michelmoore *et al.*, *Proc. Natl Acad. Sci. (USA)* 88, 9828-9832, 1991. In the case of F2 mapping populations, F2 plants are used to determine genotype, and F2 families to determine
30 phenotype. Recombinant inbred lines are produced by single-seed descent. Recombinant inbred lines, such as, for example, the F9 RILs of *A. thaliana* (eg. Lister

and Dean, *Plant J.*, 4, 745-750, 1993) will be known to those skilled in the art. Near isogenic lines (NILs) are used for fine mapping, and to determine the effect of a particular locus on transpiration efficiency. An advantage of recombinant inbred lines and double haploid lines is that they are permanent populations, and as a consequence, provide for replication of the contribution of a particular locus to the transpiration efficiency phenotype.

As for statistical methods, Single Marker Analysis (Point Analysis) is used to detect a locus in the vicinity of a single genetic marker. The mean transpiration efficiencies of a population of plants segregating for a particular marker, are compared according to the marker class. The difference between two mean transpiration efficiencies provides an estimate of the phenotypic effect of substituting one allele for another allele at the locus. To determine whether or not the inferred phenotypic effect is significantly different from zero, a simple statistical test, such as t-test or F-test, is used. A significant value indicates that a locus is located in the vicinity of the marker. Single point analysis does not require a complete molecular linkage map. The further the locus is from the marker, the less likely it is to be detected statistically, as a consequence of recombination between the marker and the gene.

In the Anova, t-test or GLM approach, the association between marker genotype and transpiration efficiency phenotype comprises:

- (i) classifying progeny of a segregating population of plants by marker genotype, such as for example, using RFLP, AFLP, SSCP, or microsatellite analyses, thereby establishing classes of plants;
- (ii) comparing the mean transpiration efficiencies of classes of plants in the segregating population, using a t-test, GLM or ANOVA; and
- (iii) determining the significance of the differences in the mean at (ii), wherein a significant difference indicates that the marker is linked to the locus for transpiration efficiency.

As will be known to those skilled in the art, the difference between the means of the classes provides an estimate of the effect of the locus in determining the transpiration efficiency of a class.

- 5 In the regression approach, the association between marker genotype and phenotype is determined by a process comprising:
- (i) assigning numeric codes to marker genotypes; and
 - (ii) determining the regression value r for transpiration efficiency against the codes, wherein a significant value for r indicates that the marker is linked to the locus
- 10 for transpiration efficiency, and wherein the regression slope gives an estimate of the effect of a particular locus on transpiration efficiency.

For QTL interval mapping, the Mapmaker algorithm developed by Lincoln *et al.*, Constructing genetic linkage maps with MAPMAKER/EXP version 3.0: A tutorial and
15 reference manual. Whitehead Institute for Biomedical Research, Cambridge, MA, USA, 1993, can be used. The principle behind interval mapping is to test a model for the presence of a QTL at many positions between two mapped marker loci. This model is a fit of a presumptive QTL to transpiration efficiency, wherein the suitability of the fit is tested by determining the maximum likelihood that a QTL for transpiration
20 efficiency lies between two segregating markers. For example, in the case of a QTL located between two segregating markers, the 2-loci marker genotypes of segregating progeny will each contain mixtures of QTL genotypes. Accordingly, it is possible to search for loci parameters that best approximate the distribution in transpiration efficiency for each marker class. Models are evaluated by computing the likelihood of
25 the observed distributions with and without fitting a QTL effect. The map position of a QTL is determined as the maximum likelihood from the distribution of likelihood values (LOD scores: ratio of likelihood that the effect occurs by linkage: likelihood that the effect occurs by chance), calculated for each locus.

- 30 Interval mapping by regression (Haley and Knott., *Heredity* 69, 315-324, 1992) is a simplification of the maximum likelihood method *supra* wherein basic QTL analysis or

regression on coded marker genotypes is performed, except that phenotypes are regressed on the probability of a QTL genotype as determined from the linkage between transpiration efficiency and the nearest flanking markers. In most cases, regression mapping gives estimates of QTL position and effect that are almost identical
5 to those given by the maximum likelihood method. The approximation deviates only at places where there are large gaps, or many missing genotypes.

In the composite interval mapping (CIM) method (Jansen and Stam, *Genetics* 136, 1447-1455, 1994; Utz and Melchinger, 1994, *supra*; Zeng, *Genetics* 136, 1457-1468,
10 1994), the analysis is performed in the usual way, except that the variance from other QTLs is accounted for by including partial regression giving more power and precision than simple interval mapping, because the effects of other QTLs are not present as residual variance. CIM can remove the bias that can be caused by the QTLs that are linked to the position being tested.

15

Publicly available software are used to map a locus for transpiration efficiency. Such software include, for example, the following:

- (i) MapMaker/QTL (<ftp://genome.wi.mit.edu/pub/mapmaker3/>), for analyzing F2 or backcross data using standard interval mapping;
- 20 (ii) MQTL, for composite interval mapping in multiple environments or for performing simple interval mapping using homozygous progeny (eg. double haploids, or recombinant inbred lines);
- (iii) PLABQTL (Utz and Melchinger, PLABlocus Version 1.0. A computer program to map QTL, Institut für Pflanzenzüchtung, Saatgutforschung und
25 Populationsgenetik, Universität Hohenheim, 70593 Stuttgart, Germany, 1995; <http://www.uni-hohenheim.de/~ipspwww/soft.html>) for composite interval mapping and simple interval mapping of a locus in mapping populations derived from a bi-parental cross by selfing, or in double haploids;
- (iv) QTL Cartographer (<http://statgen.mcsu.edu/qtlcart/cartographer.html>) for single-
30 marker regression, interval mapping, or composite interval mapping, using F2 or backcross populations;

- (v) MapQTL (<http://www.cpro.dlo.nl/cbw/>); Qgene for performing either single-marker regression or interval regression to map loci; and
 - (vi) SAS for detecting a locus by identifying associations between marker genotype and transpiration efficiency by a single marker analysis approach such as
- 5 ANOVA, t-test, GLM or REG.

In a particularly preferred embodiment, QTL cartographer or MQTL is used to identify a locus associated with the transpiration efficiency of plants.

- 10 Those skilled in the art will also be aware that it is possible to detect multiple interacting alleles or genes for a particular trait, such as, for example, using composite interval mapping approaches. To achieve this end, the composite interval mapping may be repeated to look for additional loci. Alternatively, or in addition, two or more distinct regions of the genome can be nominated as candidate loci, and a gamete
- 15 relationship matrix constructed for each candidate locus, and a 2-locus regression performed for each pair of loci, determining a best fit for the interacting effects between the two loci or alleles at those loci, including any dominance or additive effects. The algorithm described by Carlborg *et al.*, *Genetics* (2000) can be used for simultaneous mapping. In the present context, such an analysis is performed with
- 20 reference to the segregation of transpiration efficiency phenotypes in the segregating population.

Use of the ERECTA locus to enhance transpiration efficiency of plants

- As will be known to those skilled in the art, a single locus, if present in the genome of a
- 25 plant, can have a significant influence on the phenotype of the plant. For example, Grandillo *et al.*, *Theor. Appl. Genet.* 99, 978-987, 1999, showed that for tomato a selection made from a total 28 loci determining fruit size and weight explained 20% of the total phenotypic variance in this trait.

- 30 Accordingly, a second aspect of the invention provides a method of selecting a plant having enhanced transpiration efficiency, comprising:

- (a) identifying a locus associated with genetic variation in transpiration efficiency in a plant; and
- (b) selecting a plant that comprises or expresses a gene that maps to the locus.

5 By "enhanced transpiration efficiency" is meant that the plant loses less water per unit of dry matter produced, or alternatively, produces an enhanced amount of dry matter per unit of water transpired, or alternatively, fixes an increased amount of carbon per unit water transpired, relative to a counterpart plant. By "counterpart plant" is meant a plant having a similar or near-identical genetic background, such as, for example, a
10 near-isogenic plant, a sibling, or parent.

In accordance with this aspect of the invention, a locus is identified by conventional locus mapping means, and/or by homology searching for genes that map to the *ERECTA* locus on chromosome 2 of the *A. thaliana* genome, such as, for example, by
15 searching for *ERECTA* alleles or *erecta* alleles from a variety of plants, such as, for example, rice, wheat, sorghum, and maize, as described herein above.

Preferably, to select a plant that comprises or expresses the appropriate gene, marker-assisted selection (MAS) is used. As will be known to those skilled in the art, once a
20 particular locus has been identified, genetic or physical markers that are linked to the locus can be readily identified and used to confirm the presence of the locus in breeding populations. For a locus that is flanked by two tightly-linked markers that recombine only at a low frequency, the presence of the flanking markers is indicative of the presence of the locus.

25

For marker-assisted selection, it is preferred that the marker is a genetic marker (eg. a gene or allele), or a physical marker (eg. leaf hairiness or pod shape), or a molecular marker such as, for example, a restriction fragment length polymorphism (RFLP), a restriction (RAPD), amplified fragment length polymorphism (AFLP), or a short
30 sequence repeat (SSR) such as a microsatellite, or SNP. It is also within the scope of the invention to utilize any hybridization probe or amplification primer comprising at

least about 10 nucleotides in length derived from a chromosome region that is linked in the genome of a plant to an *ERECTA* locus, as a marker to select plants. Those skilled in the art will readily be able to determine such probes or primers based upon the disclosure herein, particularly for those plant genomes which may have sufficient
5 chromosome sequence in the region of interest in the genome (eg. *A. thaliana*, rice, cotton, barley, wheat, sorghum, maize, tomato, etc).

For flanking markers that are not tightly linked, such that there is a large recombination distance there between, the presence of the appropriate gene is assessed by identifying
10 those plants having both flanking markers and then selecting from those plants having an enhanced transpiration efficiency. Naturally, the greater the distance between two markers, the larger the population of plants required to identify a plant having both markers, the intervening locus and a gene within said locus. Those skilled in the art will readily be able to determine the population size required to identify a plant having a
15 particular transpiration efficiency, based upon the recombination units (cM) between two markers.

Transpiration efficiency is determined by any means known to the skilled artisan. Preferably, transpiration efficiency is determined by measuring dry matter
20 accumulation in the plant by gravimetric means, or by measuring water loss, or the ratio of CO₂ assimilation rate to stomatal conductance.

In a particularly preferred embodiment, the transpiration efficiency is determined directly, by measuring the ratio of carbon fixed (carbon assimilation rate) to water loss
25 (transpiration rate).

In an alternative embodiment, transpiration efficiency is determined indirectly from the carbon isotope discrimination value (Δ). Farquhar *et al.*, *Aust. J. Plant Physiol.* 9, 121-137, 1982, showed that carbon isotope discrimination (Δ ; a measure of the extent to
30 which the ¹³C/¹²C ratio of organic matter is less than that of CO₂ in the source air), is an effective indirect measure of transpiration efficiency. Discrimination, (Δ), is

approximately the isotope ratio of carbon in source CO₂ minus that of plant organic carbon. In a particular experiment, the source CO₂ is common to all genotypes. The determination of transpiration efficiency in this manner is based upon the constancy of the atmospheric ¹³C: ¹²C ratio (about 1.1:98.9) and the finding that ribulose
5 bisphosphate carboxylase (Rubisco) enzymes discriminate against the use of ¹³C. Thus, in C₃ plants ¹³CO₂ is less efficiently assimilated than ¹²CO₂, and the ¹³CO₂ left behind tends to diffuse back through stomata in and out of the leaf. However, when the stomata become nearly closed, the relative back-diffusion of ¹³CO₂ is more difficult to achieve and the relative intracellular concentrations of ¹³CO₂ increases, thereby
10 increasing the proportion of this isotope that is incorporated into 3-phosphoglycerate, and subsequently into dry matter. As a consequence, carbon isotope discrimination (Δ) is greatest when the overall CO₂ assimilation rate during photosynthesis (A) is small, and stomatal conductance (g_w) to water vapor is large. This relationship is represented by the following algorithm:

$$15 \quad \Delta (\text{‰}) = 27 - 36A / (g_w \times C_a)$$

wherein C_a is the ambient CO₂ concentration (ie. [¹²CO₂+¹³CO₂]). Discrimination, Δ , is approximately the isotope composition of source CO₂ minus that of plant organic carbon.

20 For a C₃ plant that exhibits a value in the range of about 4.5 ‰ to about 6.7 ‰ for the term 36A/(g_w × C_a), a 1 ‰ change in carbon isotope discrimination (Δ) corresponds to a change in transpiration efficiency in the range of about 22% to about 15%, respectively.

The negative relationship between carbon isotope discrimination (Δ) and transpiration
25 efficiency has been established for many C₃ plant species, including wheat (Farquhar and Richards, *Aust. J. Plant Physiol.* 11, 539-552, 1984; Farquhar *et al.*, *Ann. Rev. Plant Physiol.* 40, 388-397, 1989), *Stylosanthes* (Thumma *et al.*, *Proc. 9th Aust. Agronomy Conf.*, Wagga Wagga New South Wales, Australia, 1998), cotton, barley, and rice. Accordingly, a lower carbon isotope discrimination (Δ) value for a test plant
30 relative to a counterpart plant is indicative of enhanced transpiration efficiency.

In C₄ species, like maize, coefficients in the equation above are different (Farquhar 1983, Australian Journal of Plant Physiology, 10:205-226; Henderson et al., 1992, Aust. J. Plant Physiol. 19: 263-285) :

$$\Delta (\text{‰}) = 1 + 5A / (g_w \times C_a).$$

- 5 A 1‰ difference in Δ corresponds to about 38% difference in transpiration efficiency. The relationship between Δ and transpiration efficiency is positive. ¹³C preferentially accumulates in bicarbonate, the substrate for PEP carboxylation, and so discrimination against ¹³C is least when A is small and g_w is large. However, as CO₂ leakiness from the bundle sheath increases, C₄ plants behave more like C₃ plants.

10

Alternatively, or in addition, transpiration efficiency is determined by another indicator, such as, for example, leaf temperature, ash content, mineral content, or specific leaf weight (dry matter per unit leaf area). For example, specific leaf weight is positively
15 correlated with transpiration efficiency in peanuts and other species (Virgona *et al.*, *Aust. J. Plant Physiol.*, 17, 207-214, 1990; Wright *et al.*, *Crop Sci* 34, 92-97, 1994). Accordingly, a higher specific leaf weight or higher carbon gain rate for a test plant relative to a counterpart plant is indicative of enhanced transpiration efficiency of the test plant.

20

The presence of the locus can be established by hybridizing a probe or primer that is linked to an *ERECTA* locus, such as, for example, a probe or primer that hybridizes to the identified chromosome 2 region of *A. thaliana* or the identified chromosome 6 region of rice.

25

Preferably, the presence of the locus is established by hybridizing a probe or primer derived from any one or more of SEQ ID Nos: 1, 3, 5, 7, 9, 11 to 19 or 21 to 44 or from a homologous gene in another plant, or a complementary sequence to such a sequence, to genomic DNA from the plant, and detecting the hybridization using a detection means.

30

In one embodiment, detection of the hybridization is performed preferably by labelling a probe with a reporter molecule capable of producing an identifiable signal, prior to hybridization, and then detecting the signal after hybridization. Preferred reporter molecules include radioactively-labelled nucleotide triphosphates and biotinylated molecules. Preferably, variants of the genes exemplified herein, including genomic equivalents, are isolated by hybridisation under moderate stringency or more preferably, under high stringency conditions, to the probe.

Alternatively, or in addition, hybridization may be detected using any format of the polymerase chain reaction (PCR), including AFLP. For PCR, two non-complementary nucleic acid primer molecules comprising at least about 20 nucleotides in length, and more preferably at least 30 nucleotides in length are hybridized to different strands of a nucleic acid template molecule, and specific nucleic acid molecule copies of the template are amplified enzymatically. Several formats of PCR are described in McPherson *et al.*, *In: PCR A Practical Approach*, IRL Press, Oxford University Press, Oxford, United Kingdom, 1991, which is incorporated herein by reference.

For enhancing the transpiration efficiency of a plant wherein the locus is polymorphic, such as, for example, an allele, the method *supra* is modified to include the detection of the specific allele(s) linked to the desired enhancement. According to this embodiment, there is provided a method of selecting a plant having enhanced transpiration efficiency, comprising:

- (d) identifying a locus associated with genetic variation in transpiration efficiency in a plant;
- (e) identifying a polymorphic marker within said locus that is linked to enhanced transpiration efficiency; and
- (f) selecting a plant that comprises or expresses the marker.

Standard means known to the skilled artisan are used to identify a marker within the locus that is linked to enhanced transpiration efficiency. A population of plants that is segregating for the polymorphic marker is generally used, wherein the transpiration

efficiency phenotype of plants is then correlated or associated with the presence of a particular allelic form of the marker. As exemplified herein, near-isogenic or recombinant inbred lines of plants are screened to segregate alleles at the *ERECTA* locus and to correlate enhanced transpiration efficiency with the presence of the

5 *ERECTA* allele as opposed to an *erecta* allele. Alternatively, mutations are introduced into an *ERECTA* allele such as, for example, by transposon mutagenesis, chemical mutagenesis or irradiation of plant material, and mutant lines of plants are established and screened to segregate alleles at the *ERECTA* locus that are correlated with the genetic variation in transpiration efficiency.

10

Suitable markers include any one or more of the markers described herein to be suitable for MAS.

Preferably, the selection of plants in accordance with these embodiments includes the

15 additional step of introducing the locus or polymorphic marker to a plant, such as, for example, by standard breeding approaches or by recombinant means. This may be carried out at the same time, or before, selecting the locus or polymorphic marker.

Recombinant means generally include introducing a gene construct comprising the

20 locus or marker into a plant cell, selecting transformed tissue and regenerating a whole plant from the transformed tissue explant. Means for introducing recombinant DNA into plant tissue or cells include, but are not limited to, transformation using CaCl_2 and variations thereof, in particular the method described by Hanahan (1983), direct DNA uptake into protoplasts (Krens *et al*, *Nature* 296, 72-74, 1982; Paszkowski *et al*,

25 *EMBO J.* 3, 2717-2722, 1984), PEG-mediated uptake to protoplasts (Armstrong *et al*, *Plant Cell Rep.* 9, 335-339, 1990) microparticle bombardment, electroporation (Fromm *et al*, *Proc. Natl. Acad. Sci. (USA)*, 82, 5824-5828, 1985), microinjection of DNA (Crossway *et al*, *Mol. Gen. Genet.* 202, 179-185, 1986), microparticle bombardment of tissue explants or cells (Christou *et al*, *Plant Physiol.* 87, 671-674, 1988; Sanford, *Part.*

30 *Sci. Technol.* 5, 27-37, 1988), vacuum-infiltration of tissue with nucleic acid, or in the case of plants, T-DNA-mediated transfer from *Agrobacterium* to the plant tissue as

described essentially by An *et al.*, *EMBO J.* 4, 277-284, 1985; Herrera-Estrella *et al.*, Herrera-Estrella *et al.*, *Nature* 303, 209-213, 1983; Herrera-Estrella *et al.*, *EMBO J.* 2, 987-995, 1983; or Herrera-Estrella *et al.*, *In: Plant Genetic Engineering*, Cambridge University Press, N.Y., pp 63-93, 1985.

5

For microparticle bombardment of cells, a microparticle is propelled into a cell to produce a transformed cell. Any suitable ballistic cell transformation methodology and apparatus can be used in performing the present invention. Exemplary apparatus and procedures are disclosed by Stomp *et al.* (U.S. Patent No. 5,122,466) and Sanford and
10 Wolf (U.S. Patent No. 4,945,050). When using ballistic transformation procedures, the gene construct may incorporate a plasmid capable of replicating in the cell to be transformed.

Examples of microparticles suitable for use in such systems include 1 to 5 micron gold
15 spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

A whole plant may be regenerated from the transformed or transfected cell, in accordance with procedures well known in the art. Plant tissue capable of subsequent
20 clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a gene construct of the present invention and a whole plant regenerated therefrom. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls,
25 megagametophytes, callus tissue, existing meristematic tissue (eg., apical meristem, axillary buds, and root meristems), and induced meristem tissue (eg., cotyledon meristem and hypocotyl meristem).

The term "organogenesis", as used herein, means a process by which shoots and roots
30 are developed sequentially from meristematic centres.

The term "embryogenesis", as used herein, means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes.

5

The generated transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, a first generation (or T1) transformed plant may be selfed to give homozygous second generation (or T2) transformant, and the T2 plants further propagated through classical breeding
10 techniques.

The generated transformed organisms contemplated herein may take a variety of forms. For example, they may be chimeras of transformed cells and non-transformed cells; clonal transformants (eg., all cells transformed to contain the expression cassette);
15 grafts of transformed and untransformed tissues (eg., in plants, a transformed root stock grafted to an untransformed scion).

Alternatively, the transformed plants are produced by an *in planta* transformation method using *Agrobacterium tumefaciens*, such as, for example, the method described
20 by Bechtold *et al.*, *CR Acad. Sci. (Paris, Sciences de la vie/ Life Sciences)* 316, 1194-1199, 1993 or Clough *et al.*, *Plant J* 16: 735-74, 1998, wherein *A. tumefaciens* is applied to the outside of the developing flower bud and the binary vector DNA is then introduced to the developing microspore and/or macrospore and/or the developing seed, so as to produce a transformed seed. Those skilled in the art will be aware that the
25 selection of tissue for use in such a procedure may vary, however it is preferable generally to use plant material at the zygote formation stage for *in planta* transformation procedures.

30

Identification of genes for determining the transpiration efficiency of a plant

As exemplified herein, the inventors also identified specific genes or alleles that are linked to the *ERECTA* locus of *A. thaliana*, and rice and determine the transpiration efficiencies of those plants. More particularly, the transpiration efficiencies of near-
5 isogenic lines, each carrying a mutation within an *ERECTA* locus, and a correlation between transpiration efficiency phenotype and *ERECTA* expression or gene copy number are determined, thereby providing the genetic contribution of genes or alleles at the *ERECTA* locus to transpiration efficiency. This analysis permits an assessment of the genetic contribution of particular alleles to transpiration efficiency, thereby
10 determining allelic variants that are linked to a particular transpiration efficiency. Thus, the elucidation of the *ERECTA* locus for transpiration efficiency in plants facilitates the fine mapping and determination of allelic variants that modulate transpiration efficiency. The methods described herein can be applied to an assessment of the contribution of specific alleles to the transpiration efficiency phenotype for any
15 plant species that is amenable to mutagenesis such as, for example, by transposon mutagenesis, irradiation, or chemical means known to the skilled artisan for mutating plants.

Accordingly, a third aspect of the invention provides a method of identifying a gene
20 that determines the transpiration efficiency of a plant comprising:

- (a) identifying a locus associated with genetic variation in transpiration efficiency in a plant;
- (b) identifying a gene or allele that is linked to said locus, wherein said gene or allele is a candidate gene or allele for determining the transpiration efficiency of
25 a plant; and
- (c) determining the transpiration efficiencies of a panel of plants, wherein not all members of said panel comprise or express said gene or allele, and wherein variation in transpiration efficiency between the members of said panel indicates that said gene is involved in determining transpiration efficiency.

In another embodiment, the method comprises:

- (a) identifying a locus associated with genetic variation in transpiration efficiency in a plant;
- (b) identifying multiple alleles of a gene that is linked to said locus, wherein said
5 gene is a candidate gene involved for determining the transpiration efficiency of a plant; and
- (c) determining the transpiration efficiencies of a panel of plants, wherein each member of said panel comprises, and preferably expresses, at least one of said multiple alleles, wherein variation in transpiration efficiency between the
10 members of said panel indicates that said gene is involved in determining transpiration efficiency.

In the present context, the term "near isogenic plants" shall be taken to mean a population of plants having identity over a substantial proportion of their genomes,
15 notwithstanding the presence of sufficiently few differences to permit the contribution of a distinct allele or gene to the transpiration efficiency of a plant to be determined by a comparison of the transpiration efficiency phenotypes of the population. As will be known to the skilled artisan, recombinant inbred lines, lines produced by introgression of a gene or transposon followed by several generations of backcrossing, or siblings,
20 are suitable near-isogenic lines for the present purpose.

Preferably, the identified gene or allele identified by the method described in the preceding paragraph is selected from the group consisting of *ERECTA* allele, *erecta* allele, and homologs of *ERECTA*, wherein said homologs are from plants species other
25 than *A. thaliana*.

In a particularly preferred embodiment, the identified gene or allele will comprise a nucleotide sequence selected from the group consisting of:

- (d) a sequence having at least about 55% identity to a sequence selected from the
30 group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID

- NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19 SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32 SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38; SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43 and SEQ ID NO: 44;;
- 5
- (e) a sequence encoding an amino acid sequence having at least about 55% identity to an amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 20 and SEQ ID NO: 45; and
- 10
- (f) a sequence complementary to (a) or (b).

Preferably, the percentage identity is at least about 59-61% or 70% or 80%, more preferably at least about 90%, and even more preferably at least about 95% or 99%. In a particularly preferred embodiment, the identified gene or allele comprises a nucleotide sequence selected from the group consisting of:

15

- (a) a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19 SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32 SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38; SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43 and SEQ ID NO: 44;
- 20
- (b) a sequence encoding an amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 20 and SEQ ID NO: 45; and
- 25
- (c) a sequence complementary to (a) or (b).
- 30

Enhancement of transpiration efficiency using isolated genes

The identified gene or alleles, including any homologs from a plant other than *A. thaliana*, such as, for example, the wild-type *ERECTA* allele, or a homolog thereof, is useful for the production of novel plants. Such plants are produced, for example, using
5 recombinant techniques, or traditional plant breeding approaches such as by introgression.

Accordingly, a fourth aspect of the present invention provides a method of enhancing the transpiration efficiency of a plant comprising ectopically expressing in a plant an
10 isolated *ERECTA* gene or an allelic variant thereof or the protein-encoding region thereof.

Preferably, the *ERECTA* gene or allelic variant comprises a nucleotide sequence that is homologous to a protein-encoding region of a gene that is linked to the *A. thaliana*
15 *ERECTA* locus on chromosome 2.

In a particularly preferred embodiment, the isolated gene comprises a nucleotide sequence selected from the group consisting of:

- (a) a sequence having at least about 55% identity to a sequence selected from the
20 group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28,
25 SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43 and SEQ ID NO: 44;
- (b) a sequence encoding an amino acid sequence having at least about 55% identity
30 to an amino acid sequence selected from the group consisting of SEQ ID NO: 2,

SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 20 and SEQ ID NO: 45; and

(c) a sequence complementary to (a) or (b).

- 5 Preferably, the percentage identity is at least about 59-61% or 70% or 80%, more preferably at least about 90%, and even more preferably at least about 95% or 99%.

In a particularly preferred embodiment, the isolated gene or allele comprises a nucleotide sequence selected from the group consisting of:

- 10 (a) a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43 and SEQ ID NO: 44;
- 15 (b) a sequence encoding an amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 20 and SEQ ID NO: 45; and
- 20 (c) a sequence complementary to (a) or (b).

To ectopically express the isolated gene in a plant, the protein-encoding portion of the gene is generally placed in operable connection with a promoter sequence that is operable in the plant, which may be the endogenous promoter or alternatively, a heterologous promoter, and a transcription termination sequence, which also may be the endogenous or an heterologous sequence relative to the gene of interest. The promoter and protein-encoding portion and transcription termination sequence are generally provided in the form of a gene construct, to facilitate introduction and maintenance of the gene in a plant where it is to be ectopically expressed. Numerous

25

30

vectors suitable for introducing genes into plants have been described and are readily available. These may be adapted for expressing an isolated gene in a plant to enhance transpiration efficiency therein.

- 5 Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a classical eukaryotic genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (ie. upstream activating sequences, enhancers and silencers) which alter gene expression in response to
- 10 developmental and/or external stimuli, or in a tissue-specific manner. In the present context, the term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of said sense molecule in a cell. Preferred promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression and/or to alter the spatial expression
- 15 and/or temporal expression of a nucleic acid molecule to which it is operably connected. For example, copper-responsive regulatory elements may be placed adjacent to a heterologous promoter sequence driving expression of a nucleic acid molecule to confer copper inducible expression thereon.
- 20 Placing a nucleic acid molecule under the regulatory control of a promoter sequence means positioning said molecule such that expression is controlled by the promoter sequence. A promoter is usually, but not necessarily, positioned upstream or 5' of the protein-encoding portion of the gene that it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of
- 25 transcription of the structural protein-encoding nucleotide sequences, or a chimeric gene comprising same. In the construction of heterologous promoter/structural gene combinations it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting, ie., the gene from which the
- 30 promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning

of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting, ie., the genes from which it is derived. Again, as is known in the art, some variation in this distance can also occur.

5

Promoters suitable for use in gene constructs of the present invention include those promoters derived from the genes of viruses, yeasts, moulds, bacteria, insects, birds, mammals and plants which are capable of functioning in plant cells, including monocotyledonous or dicotyledonous plants, or tissues or organs derived from such
10 cells. The promoter may regulate gene expression constitutively, or differentially with respect to the tissue in which expression occurs or, with respect to the developmental stage at which expression occurs, or in response to external stimuli such as physiological stresses, pathogens, or metal ions, amongst others.

15 Examples of promoters useful in performing this embodiment include the CaMV 35S promoter, rice actin promoter, rice actin promoter linked to rice actin intron (PAR-IAR) (McElroy *et al*, *Mol and Gen Genetics*, 231(1), 150-160, 1991), *NOS* promoter, octopine synthase (OCS) promoter, *Arabidopsis thaliana SSU* gene promoter, napin seed-specific promoter, PcSVMV, promoters capable of inducing expression under
20 hydric stress, as described by, for example, Kasuga *et al*, *Nature Biotechnology*, 17, 287-291, 1999), SCSV promoter, SCBV promoter, 35s promoter (Kay *et al*, *Science* 236, 4805, 1987) and the like. In addition to the specific promoters identified herein, cellular promoters for so-called housekeeping genes, including the actin promoters, or promoters of histone-encoding genes, are useful.

25

The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. Terminators are 3'-non-translated DNA sequences containing a polyadenylation signal, that facilitate the addition of a polyadenylate sequence to the 3'-end of a primary transcript. Terminators active in
30 cells derived from viruses, yeasts, moulds, bacteria, insects, birds, mammals and plants

are known and described in the literature. They are isolatable from bacteria, fungi, viruses, animals and/or plants.

Examples of terminators particularly suitable for use in the gene constructs of the present invention include the nopaline synthase (NOS) gene terminator of *Agrobacterium tumefaciens*, the terminator of the Cauliflower mosaic virus (CaMV) 35S gene, the *zein* gene terminator from *Zea mays*, the Rubisco small subunit (SSU) gene terminator sequences and subclover stunt virus (SCSV) gene sequence terminators, amongst others.

10

Those skilled in the art will be aware of additional promoter sequences and terminator sequences that may be suitable for use in performing the invention. Such sequences may readily be used without any undue experimentation.

15 Preferably, the gene construct further comprises an origin of replication sequence for its replication in a specific cell type, for example a bacterial cell, when said gene construct is required to be maintained as an episomal genetic element (eg. plasmid or cosmid molecule) in said cell. Preferred origins of replication include, but are not limited to, the *f1*-ori and *colE1* origins of replication.

20

Preferably, the gene construct further comprises a selectable marker gene or genes that are functional in a cell into which said gene construct is introduced.

As used herein, the term "selectable marker gene" includes any gene which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection of cells which are transfected or transformed with a gene construct of the invention or a derivative thereof.

Suitable selectable marker genes contemplated herein include the ampicillin resistance (Amp^r), tetracyclin-resistance gene (Tc^r), bacterial kanamycin resistance gene (Kan^r), phosphinothricin resistance gene, neomycin phosphotransferase gene (*nptII*),

30

hygromycin resistance gene, gentamycin resistance gene (*gent*), β -glucuronidase (GUS) gene, chloramphenicol acetyltransferase (CAT) gene, and luciferase gene, Green Fluorescent Protein gene (EGFP and variants), amongst others.

- 5 In a related embodiment, the invention extends to the use of an isolated gene comprising a nucleotide sequence that is homologous to a protein-encoding region of a gene of *A. thaliana* that is positioned between about 46cM to about 50.74cM on chromosome 2 in the preparation of a gene construct for enhancing the transpiration efficiency of a plant.

10

- In an alternative embodiment of the invention, the transpiration efficiency of a plant is enhanced by classical breeding approaches, comprising introgressing the isolated gene into a plant. For introgression of a gene, the gene is transferred from its native genetic background into another genetic background using standard breeding, for example, a
15 gene that enhances transpiration efficiency in a progenitor such as a diploid cotton or diploid wheat may be transferred into a commercial tetraploid cotton or hexaploid wheat, respectively, by standard crossing, followed by several generations of back-crossing to remove the genetic background of the progenitor. Naturally, continued selection of the gene of interest is required, such as, for example, facilitated by the use
20 of markers.

A further aspect of the present invention provides a plant having enhanced transpiration efficiency, wherein said plant is produced by a method described herein.

- 25 Clearly the ERECTA genes, allelic variants and protein coding regions described herein are useful in determining other proteins that are involved in the transpiration process in plants. For example, an ERECTA gene, allelic variant thereof or protein coding region thereof may be used in a forward 'n'-hybrid assay to determine if said peptide is able to bind to a protein or peptide of interest. Forward 'n' hybrid methods
30 are well known in the art, and are described for example, by Vidal and Legrain *Nucl. Acid Res.* 27(4), 919-929 (1999) and references therein, and include yeast two- hybrid,

bacterial two-hybrid, mammalian two-hybrid, PolIII (two) hybrid, the Tribid system, the ubiquitin based split protein sensor system and the SOS recruitment system. Such methods are incorporated herein by reference

5 In adapting a standard forward two-hybrid assay to the present invention, an ERECTA protein is expressed as a fusion protein with a DNA binding domain from, for example, the yeast GAL4 protein. Methods of constructing expression constructs for the expression of such fusion proteins are well known in the art, and are described, for example, in Sambrook et al (In: Molecular Cloning: A laboratory Manual, Cold Spring
10 Harbour, New York, Second Edition, 1989). A second fusion protein is also expressed in the yeast all, said fusion protein comprising, for example, a protein thought to interact with an ERECTA protein, for example the GAL4 activation domain. These two constructs are then expressed in a yeast cell in which, a reporter molecule (e.g., *tef*, *Amp^r*, *Rif^r*, *bsdf^r*, *zeof^r*, *Kan^r*, *gfp*, *cobA*, *LacZ*, *TRP1*, *LYS2*, *HIS3*, *HIS5*, *LEU2*, *URA3*,
15 *ADE2*, *MET13*, *MET15*) under the control of a minimal promoter placed in operable connection with a GAL 4 binding site. If the proteins do not interact, a reporter molecule is not expressed. However, if said proteins do interact, said reporter molecule is expressed. Accordingly a protein, polypeptide, peptide that is able to specifically bind a target protein is identified.

20

A forward 'n'-hybrid method may be modified to facilitate high throughput screening of a library of peptides, polypeptides and/or proteins in order to determine those that interact with an ERECTA protein. Methods of screening libraries of proteins are well known in the art and are described, for example, in Scopes (In: Protein Purification:
25 Principles and Practice, Third Edition, Springer Verlag, 1994). Proteins identified by this method are potentially involved in the transpiration process in plants.

The present invention is further described with reference to the following non-limiting examples.

30

EXAMPLE 1

$^{12}\text{C}/^{13}\text{C}$ discrimination as a marker for screening genetic variation
in transpiration efficiency.

Experimental conditions and sampling procedures were established to allow the control
5 of many factors, other than genetic, that influence transpiration efficiency at the level of
individual leaves and plants. These factors fall into several categories: (a)
characteristics of the seedling's micro-environment: temperature, light, humidity,
boundary layer around the leaves, root growth conditions; (b) developmental and
morphological effects that modify gas exchange and C metabolism and therefore
10 carbon isotopic signature (eg age, stage, posture); and (c) seed effects.

We developed high resolution mass-spectrometer techniques for measuring C isotope
ratios in whole tissues or carbon compounds such as soluble sugars -ie a measure of
integrated transpiration efficiency over the plant's life or over a day, respectively, and
15 also for measuring instantaneous transpiration efficiency during gas exchange.

This means:

- 0.1 per mil analytical precision in the measurement of the isotopic composition
of leaf carbon. Discrimination, (Δ), is approximately the isotope ratio of carbon
20 in source CO_2 minus that of plant organic carbon. In a particular experiment, the
source CO_2 is common to all genotypes.
- 0.1 per mil biological precision, that is variation between replicated seedlings,
grown in soil, either in growth chambers or in glasshouses with CO_2 , humidity
and temperature control (corresponding to approximately 1.5% variation in
25 transpiration efficiency).
- The ability to grow and screen large batches of seedlings in glasshouses or
growth chambers (up to 1500), under standardised leaf and root growth
conditions, to a rosette size of several cm within 2-3 weeks allowing individual
measurements, on the same plant, of isotope ratios and also of the underlying
30 properties (eg in situ measurement of leaf temperature by infra-red thermometry

as a measure of stomatal conductance; chlorophyll fluorescence; leaf expansion).

EXAMPLE 2

5 Natural genetic variation in transpiration efficiency in *Arabidopsis thaliana*
A. thaliana ecotypes were screened for leaf Δ under glasshouse conditions. There was a large spread of values (corresponding to approximately 30 % genetic variation in transpiration efficiency). However, large environmental effects were noted. A few contrasted ecotypes were selected at the two extremes of the range of Δ values and
10 compared under various conditions of irradiance (150 to 500 $\mu\text{E m}^{-2}\text{s}^{-1}$), light spectrum (Red/Far-Red ratios) and air humidity (60 to 90%) while roots were always well watered. The magnitude of genetic differences in transpiration efficiency was very much influenced by environmental conditions. This was in part due to variations among ecotypes in the dependence of photosynthesis on light and vapour pressure deficit.
15 Genetic differences were maximal under a combination of high light and low humidity, in growth chambers.

The ecotypes *Columbia (Col)* and *Landsberg erecta (Ld-er)* have extreme carbon isotope discrimination values, with *Col* always having smaller Δ values than *Ld-er* ie
20 less negative $\delta^{13}\text{C}$ isotopic compositions, and thus a greater transpiration efficiency.

EXAMPLE 3

Identification of a locus associated with transpiration efficiency in *A. thaliana*

25 Quantitative Trait Loci (QTL) analysis of the Lister and Dean's (1993) Recombinant Inbred Lines (later referred to as RILs) was performed to identify and map a locus associated with carbon isotope discrimination (Δ). The RILs were from a cross between Col-4 and Ler-0. Our analysis showed the importance of genes around the *ER* locus on chr2, and a role for genes other than *ERECTA* in conferring transpiration
30 efficiency on *A. thaliana*.

More particularly, 300 RI mapping lines between Col and Ler ecotypes, available at the Arabidopsis Stock Centre, were generated from a cross between the *Arabidopsis* ecotypes Columbia (Col4) and Landsberg erecta (Ler-0 carrying *er1*) (Lister and Dean, 1993), using Columbia as the male parent. A subset of 100 of these lines, chosen as the
5 most densely and reliably mapped were used in the present analysis.

The seeds were multiplied in a glasshouse in an attempt to minimize confounding seed effects in our comparisons. Large numbers of seeds were obtained for most lines except for a few, including Col4 parent, which had to be re-ordered following low seed
10 viability of the original sample sent by the Stock Centre. The seeds harvested in these propagation runs were used throughout all our experiments to date.

Loci were analysed using two programs, QTL cartographer and MQTL. These programs compute statistics of a trait at each marker position, using a range of methods
15 [linear regression (LR), stepwise regression (SR), and likelihood approaches (Single interval mapping (SIM) which treats values at individual markers as independent values, and composite interval mapping (CIM) which allows for interactions between markers and associated locus)]. By nature each of these methods has some biases and embedded assumptions, hence the importance of analysing data with more than one
20 program. Only results that were consistent between the two programs, and robust to additions or deletions to the set of background markers used for composite interval mapping are reported below.

Initial QTL analysis was done in parallel to seed multiplication on a subset of 40 lines
25 for which enough seeds were sent. Once all seeds had been multiplied this was repeated on the full set of 100 lines. These two analyses indicated the existence of a locus for carbon isotope discrimination (Δ), that maps to the region including the *ERECTA* locus on chromosome 2, at approximately 46-51 cM (Table 1, run 1&2).

30 Given the complexity and integrative nature of Δ as a physiological trait, such a small number of loci associated with the trait was not expected. Subsequent experiments

were therefore designed to test these results and assess their stability across the range of environmental conditions known for their effects on gene expression related to Δ (see above). QTL analysis was repeated on several completely independent data sets obtained under highly controlled conditions in glasshouses or growth chambers, where
5 either air humidity, photoperiod or irradiance (amount, diurnal pattern, day to day variation) was varied. Depending on the experiment, all 100 recombinants inbred lines were included or only the subset of lines with cross-overs on chromosome 2. These experiments confirmed that genetic variation in Δ could be mostly ascribed to a portion of chromosome 2 (Table 1) between about 46-50.7 cM.

10

When RILs were sorted graphically according to carbon isotope discrimination and their genotype at the *ER* marker (50.64 cM) and its vicinity (*Ld-er1* genotype or *Col-ER* genotype), lines which were *Ld-er* at the *ERECTA* marker ranked mostly at the high end of carbon isotope discrimination values. In contrast, lines having a *Col-ERECTA*
15 marker genotype ranked mostly at the low end of carbon isotope discrimination values (data available on request). In the middle of the range of carbon isotope discrimination values, there was some overlap between the two sets of lines. Some lines were always at an extreme (in all 18 experiments performed), while the ranking of other lines was more unstable. These data indicate a locus for transpiration efficiency, as determined
20 by the carbon isotope discrimination value, in the vicinity of the *ERECTA* locus on chromosome 2 (Table 1). This locus most likely involves the *ER* gene. Depending on the positions of cross-overs between *Ld-er* and *Col*, recombination between *ERECTA* and one or more of the other genes influences the transpiration efficiency phenotype of the progeny.

25

EXAMPLE 4

Transformation protocol for maize

Gun transformation

30 A suitable method for maize transformation is based on the use of a particle gun identical to that described by J. Finer (1992, *Plant Cell Report*, 11:323-328). The target cells are fast dividing undifferentiated cells having maintained a capacity to regenerate

in whole plants. This type of cells composes the embryogenic callus (called type II) of maize. These calluses are obtained from immature embryos of genotype HiII according to the method and on medium described by Armstrong (Maize Handbook ; 1994 M. Freeling, V. Walbot Eds ; pp.665-671).

5

These fragments of the calluses having a surface from 10 to 20 mm² are arranged, 4 hour before bombardment, by putting 16 fragments by dish in the center of a Petri dish containing an culture medium identical to the medium of initiation of calluses, supplemented with 0.2 M of mannitol + 0,2 M of sorbitol. Plasmids containing the
10 ERECTA sequences to be introduced, are purified on QiagenR column following the instructions of the manufacturer.

They are then precipitated on particles of tungsten (M10) following the protocol described by Klein *et al*, *Nature*, 327, 70-73, (1987). Particles so coated are sent
15 towards the target cells by means of the gun according to the protocol described by Finer *et al*, *Plant Cell Report*, 11:323-328, 1992. The bombarded dishes of calluses are then sealed by means of ScellofraisR then cultivated in the dark at 27°C.

The first transplanting takes place 24 hours later, then every other week during 3
20 months on medium identical to the medium of initiation supplemented with a selective agent. After 3 months or sometimes earlier, one can obtain calluses the growth of which is not inhibited by the selective agent, usually and mainly consisting of cells resulting from the division of a cell having integrated into its genetic patrimony one or several copies of the gene of selection. The frequency of obtaining of such calluses is
25 about 0,8 callus by bombarded dish.

These calluses are identified, individualized, amplified then cultivated so as to regenerate seedlings, by modifying the hormonal and osmotic equilibrium of the cells according to the method described by Vain and al. (1989, *Plant Cell tissue and organ*
30 *Culture* 18:143-151). These plants are then acclimatized in greenhouse where they can be crossed for obtaining hybrids or self-fertilized.

In a preferential way, one can use a similar protocol, the principle of which is described in *Methods of Molecular Biology: Plant gene transfer and expression protocols* (1995, vol. 49, PP113-123), and in which the immature embryos of genotype HiII are directly
5 bombarded with golden particles coated with plasmides ERECTA to introduce, prepared according to the protocol described by Barcelo and Lazzeri (1995, *Methods of Molecular Biology*, 49:113-123).

Steps of transformation, selection of the events, maturation and regeneration are similar
10 to those described in the previous protocol.

Agrobacterium transformation

Another technique of useful transformation within the framework of the invention uses *Agrobacterium tumefaciens*, according to the protocol described by Ishida and al (1996,
15 *Nature Biotechnology* 14 : 754-750), in particular starting from immature embryos taken 10 days after fertilization.

All the used media are referenced in the quoted reference. The transformation begins with a phase of co-culture where the immature embryos of the maize plants are put in contact during at least 5 minutes with *Agrobacterium tumefaciens* LBA 4404
20 containing the superbinary vectors.

The superbinary plasmid is the result of an homologous recombination between an intermediate vector carrying the T-DNA, and containing the gene of interest and/or the marker gene of selection, and the vector pSB1 of Japan Tobacco (EP 672 752)
25 containing: the *virB* and *virG* genes of the plasmide pTiBo542 present in the supervirulent strain A281 of *Agrobacterium tumefaciens* (ATCC 37349) and an homologous region found in the intermediate vector, allowing homologous recombination.

30 Embryos are then placed on LSAs medium for 3 days in the dark and at 25°C. A first selection is made on the transformed calluses: embryogenic calluses are transferred on

LSD5 medium containing phosphinotricine (5 mg / l) and céfotaxime (250 mg / l) (elimination or limitation of contamination by *Agrobacterium tumefaciens*).

- This step is performed during 2 weeks in the dark and at 25°C. The second step of
- 5 selection is realized by transfer of the embryos which developed on LSD5 medium, on LSD10 medium (phosphinotricine, 10 mg / l) in the presence of céfotaxime, during 3 weeks at the same conditions as previously. The third stage of selection consists in excising the calluses of type I (fragments from 1 to 2 mm) and in transferring them for 3 weeks in the darkness and at 25°C on LSD 10 medium in the presence of céfotaxime.
- 10 The regeneration of seedlings is made by excising the calluses of type I which proliferated and by transferring them on LSZ medium in the presence of phosphinotricine (5 mg / l) and of céfotaxime for 2 weeks at 22°C and under continuous light.
- 15 Seedlings having regenerated are transferred on RM medium + G2 containing Augmentin (100mg / l) for 2 weeks at 22°C and under continuous illumination for the development step. The obtained plants are then transferred to the phytotron with the aim of acclimatizing.

20

EXAMPLE 5

Detecting expression of ERECTA protéin

Extraction of ERECTA from leaves and seeds of maize.

- Leaves are harvested and immediately frozen in liquid nitrogen. Grinding is made in a
- 25 mortar cleaned in ethanol 100 % and cooled on ice. A foliar disc of 18 mm diameter is extracted in 200 µL of extraction buffer: Tris-HCl pH 8.0, glycerol 20%, MgCl₂ 10 mM, EDTA 1 mM, DTT 1 mM, PVP insoluble 2% (p/v), Fontainebleau sand et protease inhibitors: leupeptin 2mg/L, chymostatin 2mg/L, PMSF 1mM and E64 1mg/L. The ground material is then centrifuged in 4°C during 15 minutes at 20000g to
- 30 eliminate fragments.

Grains are first reduced to powder in a bead-crusher (Retsch). Proteins are extracted by suspending 100µL of powder in 400 µL of the previously described buffer on ice. This mixture is vortexed and centrifuged at 4°C during 15 minutes et 20000g to eliminate fragments.

5

ERECTA protein levels are then measured using techniques known to those skilled in the art, and described, for example, in Scopes (*In: Protein purification: principles and practice*, Third Edition, Springer Verlag, 1994).

10

EXAMPLE 6

Determination of a role for the *ERECTA* gene in regulating transpiration efficiency

We compared Col and Ler ecotypes with near-isogenic mutant lines for the *erecta* gene, to examine a possible role of the *ERECTA* gene in determining carbon isotope discrimination (Δ).

15

Plants expressing the wild type *ERECTA* gene (SEQ ID NO: 1), or an *erecta* mutant allele in the Columbia background (eg. Col-*er1*, Col-*er2*, Col-*er101* to -*er105*; or Col-*er106* to -*er123*) and in Landsberg background (*Ld-er1*) have been publicly described.

Three of these mutants were available for comparison to the isogenic or near-isogenic lines (Table 2).

20

Col4 (ER) and *Ld-er1*, the parental lines for Lister and Dean's RILs were systematically included in the comparison. Where possible, other Col "ecotypes" were also included, (eg. Col0, Col1, Col3-7), to assess their similarity with respect to carbon isotope discrimination, especially compared to the RIL parental ecotype Col4.

25

The results of these comparisons are described in Table 3. Data indicate the differences in carbon isotope discrimination values between *er* and *ER* lines for 15 different experimental runs corresponding to growth under low to high light (100 to 800 µE m⁻²

30

s⁻¹), low to high humidity (40 to 85%), short to long days (8, 10, 24hrs), normal to high temperatures (22/20°C to 28/20 °C).

As expected, the spread of carbon isotope discrimination values among lines varied
5 with environmental conditions. Lines carrying *er* mutations have a greater carbon
isotope discrimination value overall than those having the *ER* wild type gene (see Table
3, column 1), indicative of a lower water use-efficiency. There is usually little
difference in C isotopic discrimination between the various Col lines, (see the similar
averages obtained for columns 2, 3, and 4 in Table 3, wherein *er105* is compared to 3
10 different Col ecotypes, Col0, Col4 and 3176 or Col1). When present, the *er105* mutant
always has the greatest carbon isotope discrimination value of all lines, including *er1*
and *er2* (columns 2-4 compared to columns 5-6 in Table 3, or column 8 compared to
column 9 in Table 3). The value measured in the *er105* mutant is always significantly
greater than in the *ER* isogenic line (column 4 in Table 3). The value measured in *er1*
15 (Landsberg parental line NW20) is usually also greater than that in the *ER* lines 3177
(near isogenic, column 6 of Table 3), and to a lesser extent Col4 (Columbia parental
line, column 7 of Table 3). These observations give direct evidence that the *ERECTA*
gene plays a significant role in determining genetic differences in carbon isotopic
discrimination in *Arabidopsis*.

20

This conclusion is independently confirmed by leaf gas exchange measurements that
allow the direct measure of transpiration efficiency (ratio of net CO₂ fixation to water
loss; column 4 in Table 4; Figures 1a-1c, 2a-2c). Measurements on mature leaves
reveal that *ER* lines are characterised by a greater ratio of CO₂ assimilation to water
25 loss than lines carrying *er* mutations. This is most obvious when comparing the pair
Col1/er105 with a 21% greater transpiration efficiency (ratio A/E) in *Col1* than *er105*,
or the pair *Col1/er2* with a 16% greater transpiration efficiency in *Col1*. Consistent
with the measurements of carbon isotope discrimination, the effect *er/ER* is relatively
smaller in the *Ld* background (9% greater ratio A/E in *Ld-ER* (3177) than in the *Ld-er1*
30 (NSW20) line).

Also consistent with the carbon discrimination measurements, is the 20% difference in transpiration efficiency between the two RILs parental lines (4.06 and 3.38 mmolC/molH₂O in *Col4-ER* and *Ld-er1*, respectively).

5 The fact that of all 3 *erecta* mutants examined, *er105* has the most extreme carbon discrimination and transpiration efficiency phenotypes suggests that the *er105* mutation affects a more crucial part of the *ERECTA* gene than *er2* or *er1*. This is consistent with the published data on the *er105* mutant. This mutation corresponds to the insertion of a large "foreign insert" in the *ERECTA* gene. The insertion inhibits transcription of the
10 gene and causes the strongest *erecta* phenotype of all *erecta* mutants isolated in Col (with respect to inflorescence clustering and silique width and shape). Alternatively, or in addition, data indicate that *erecta* mutations have a stronger effect on carbon isotope discrimination values in a Columbia genetic background than in a Landsberg background (comparison of phenotypic effects of *er105* and *er1*), implying that other
15 genes, polymorphic between Landsberg and Columbia ecotypes, interact with *ERECTA* in determining transpiration efficiency. This could also account for the greater difference in transpiration efficiency between *er/ER* lines in Col background than in a Ld background (see above, Table 4). Alternatively, or in addition, data indicate that the *erecta* mutation is not the only mutation present in the *er105* mutant. For example, the
20 mutagenized Col seeds may have carried the *g11* mutation, induced by the fast neutron irradiation, that also contributes to the phenotype observed.

A comparison of transcript profiles in *er/ER* isogenic lines (in both Col and Ld background) allows determination of the involvement of additional genes to *ERECTA*
25 and the effect of environment on their expression.

EXAMPLE 7

QTL detection centred on the *ERECTA* marker and *ERECTA* gene locus on chromosome 2 of *Arabidopsis thaliana*

5 1. Methods

Numerous runs using the Lister and Dean (1993) Recombinant Inbred Lines between Col-4 and Ler-0 were grown in a temperature controlled glass house (20/20°C) or within growth cabinets (21°C and light levels ranging from 100 to 500 $\mu\text{E m}^{-2} \text{s}^{-1}$ irradiance, and 50-70% relative humidity). Runs included a variety of all 100 RILs as
10 well as subsets of these 100 along with parental Col-4 and Ler-0 (NW20) parental lines. Individual RILs were replicated within runs. Seeds were either cold-treated on moist filter paper for 2-4 days, cold-treated and planted directly onto soil; or plated onto agar, cold-treated for 2-4 days, grown on agar for about 11-15 days before being transferred to soil. Plants were well-watered, and grown for 4-5 weeks before harvest. Samples
15 (whole or part of rosette) were collected and dried in an 80°C oven before being ground and analysed for C isotopic composition. The value used for QTL analysis for an individual line was the average of the replicated plants of that line within one run.

2. Marker Selection

20 The standard set of 64 markers for the Lister and Dean recombinant lines were downloaded from the NASC website. Additional markers were added to this data set when significance was first determined to get finer scale mapping in the regions of interest. A total of 121 markers were used across the 5 chromosomes.

25 3. Analysis

Runs were analysed using Simple Interval Mapping (SIM)(Lander & Botstein 1989) and Composite Interval Mapping (CIM) (Zeng 1993 & 1994). Two programs were used to analyses the data, QTL Cartographer version 1.14 (Basten et al. 1999) and MQTL version 0.98 (Tinker and Mathers 1995). The two programs differ in how they deal
30 with background markers for Composite Interval Mapping (CIM). Within MQTL the background markers are chosen at random and put into the map input file. Within QTL

Cartographer the background markers are not chosen at random but rather are chosen from the Stepwise Regression analysis selecting the "best" background markers. The setting or choosing of these markers also has an influence on the level of statistical significance. Tinker argues that it is not possible to find an appropriate threshold for statistical error control when background markers are selected based on the data. Hence we used the two programs and have concentrated on QTLs that were present in both sets of analysis.

a) QTL cartographer

Qstat was used to determine whether the data had a normal distribution (if not then measures were taken to fix the distribution). Linear Regression (LR) and Stepwise Regression (SR) were performed using the default settings (Stepwise regression used forward with backward elimination) 5% significance. Simple Interval and CIM were performed using the Zmap.qtl function. The data were analysed across all chromosomes with a walking speed of 2 cM. For model 6 (CIM) the number of background parameters was left at the default of 5 along with the window size which was left on the default of 10 cM. One thousand permutations were performed within CIM (Churchill and Doerge 1994). Eqt1 was then run to determine the significant QTLs.

20

b) MQTL

The same set of markers used in QTL Cartographer was used in MQTL. Background markers were chosen at random for CIM. The number of markers chosen was approximately half that of the number of RILs used in the set. The default setting of a walking speed of 5 cM was selected, 3000 permutations were performed to determine significance levels with type 1 error set at 5% .

QTLs that were present in both programs and from varied background marker sets from within MQTL were considered genuine. This, coupled with repeated QTL analysis across independent experiments, lead to a significant repeatable locus surrounding the ERECTA gene on Chromosome 2 (Table 5).

30

Data in Table 5 indicate that there is a major QTL with a LOD score significant at the probability level of 5% and, for most runs, of even 1%, on *Arabidopsis thaliana* chromosome 2. In all cases, that interval sits above the *ER* marker on chromosome 2.

- 5 Depending on the experimental run, this QTL explains 18 to 64% (see column R^2) of the total genetic variance in transpiration efficiency.

Data in Figure 3 indicate a positive additive effect of the identified QTL based upon the mean value of the carbon isotope composition in plants carrying the Col-4 *ERECTA*

10 allele.

EXAMPLE 8

Complementation Test: transformation of *A. thaliana* lines carrying *erecta* mutations with the wild type *ERECTA* gene under the control of the 35S promoter.

15

1. Methods

Two Columbia *erecta* lines were transformed using a binary vector generously given by Dr Keiko Torii. That plasmid was constructed using the vector plasmid pPZP222 (see details on this vector in Hajdukiewics *et al. Plant Mol Biol* 25, 989-994, 1994).

- 20 The pPZP vectors carry chimeric genes in a CaMV 35S expression cassette that confer resistance to kanamycin or gentamycin in plants. The plant selectable marker (gentamycin resistance gene for the pPZP222 vector) is cloned next to the LB. Cloning sites for the gene of interest (ER in our case) is between the plant marker and the RB sequences. This ensures that that gene is transferred to plant first, followed by the *gent* gene. Resistance to gentamycin will therefore be obtained only if the ER gene is also present.
- 25

The binary vector was transferred to disarmed strain AGL1 of *Agrobacterium tumefaciens* by standard tri-parental matings (Ditta *et al, 1980, PNAS* 77,7347-7351)

- 30 using the pRK2013 helper strain of *E coli*.

Arabidopsis plants were transformed using the standard floral dip method for transformation by disarmed strains of *A. tumefaciens* (Clough and Bent, 1998, *The Plant Journal* 16, 735-743).

5 Two Columbia *erecta* lines were transformed, for which we had numerous data showing consistently more negative isotopic values in those lines (ie lower transpiration efficiency) than in near-isogenic Col ER wild type plants. These two lines were as follows:

1. er 105, a knock-out mutant due to the insertion of a large piece of DNA in the
10 ERECTA gene and
2. line Col-er2 (3401 NASC identifier), same as er106 (Lease *et al.* 2001).

Seedlings were screened on MS plates on 100 µg/ml gentamycin sulfate. Putative transformants were transferred to soil and their progeny screened again for gentamycin
15 resistance, for confirmation and identification of homozygous lines and T3 seed collection.

Many independent transformant lines were obtained and among those were several ER homozygous lines, which were selected for subsequent analysis (see Table 6).

20

A stable transgenic homozygous Landsberg ER line also obtained by transforming the Ld-er1 ecotype (NW20) with the same construct as described above was given to us by Dr Keiko Torii (line T3-7K in Table 6 or "T2+ER" in Figures 6-9).

25 2. Results:

Initial analysis of several ER transformants in the Col-er105, Col-er106/er2, and Ld-er1 background (as shown in Table 6 above):

Effective transformation was ascertained and ER expression levels were quantified in
30 several independent T2 transformants using real-time quantitative PCR (ABI PRISM 7700, Sequence Detection System User Bulletin #2. 1997). Basically that technique

allowed us to quantify the copy number of the ER gene in lines of interest, after normalisation to the copy number of a control gene, in the same plants (same RNA pool). 18S ribosomal RNA gene was used as a control gene after checking its expression was not affected by changes in ER expression.

5

Results are shown in Figures 4a, 4b and 4c, wherein the y-axis in each figure describes the *erecta* mRNA copy number (normalised to that of 18S mRNA) in wild type ER lines, *er* mutants, and ER transgenics in both Columbia and Landsberg backgrounds.

- 10 All ER transgenic lines, except line 145 (Figure 4a) showed increased mRNA copy number: from 4 to 170 fold increase compared with the null controls. Interestingly, all lines, even those with hugely increased mRNA levels look "normal", healthy and of similar size.
- 15 Initial phenotypic analysis shows complementation of the "transpiration efficiency phenotype". In other words, *ER* transgenic lines show less negative carbon isotopic composition values than null *er* control and null lines as shown in Table 7. Those values converge towards values measured for wild type ER ecotypes. Hence in a Columbia background, ER transgenics display values of -30.6 to -31.2 per mil on
- 20 average compared to values of -31.7 to -32.2 per mil in the null transgenics (Table 7), and -30.9 per mil in the Col0 ER wild type (background ecotype for mutant *er*-105). The less negative carbon isotopic compositions in ER transgenics is indicative of greater transpiration efficiency in these plants, as expected.
- 25 The data presented in Table 7 are confirmed by direct measurement of leaf transpiration efficiency (ratio A/E of CO_2 assimilation rate per unit leaf area to transpiration rate) using gas exchange techniques. Stomatal density, leaf photosynthetic capacity and growth rate are also determined to analyze the underlying causes of the reversion of the transpiration efficiency phenotype (leaf development and anatomy, biochemical
- 30 properties of leaves, stomatal characteristics).

EXAMPLE 9

Tissue specificity in the expression of the *ERECTA* gene in wild type rice

Oryza sativa (cv Nipponbare):

- 5 An ortholog of the *A. thaliana* *ERECTA* allele (SEQ ID NO: 1) in rice was identified *in silico* by homology searching of the NCBI protein database using the BLAST programme under standard conditions. The input sequence was SEQ ID NO: 2. The nucleotide sequence of the rice ortholog is presented in SEQ ID NO: 3, with the encoded protein comprising the amino acid sequence set forth in SEQ ID NO: 4.

10

The mRNA copy number of the rice *ERECTA* gene was determined for various plant organs/parts, as indicated in Figures 5a and 5b. *ERECTA* mRNA copy numbers were determined by quantitative real-time PCR, using 18S mRNA as an internal control gene for normalization of data. The pattern of *ERECTA* expression in rice was similar to the
15 pattern of gene expression in *A. thaliana*, with highest expression observed in young meristematic tissues, young leaves and even more, the inflorescences. No or very low expression is found in roots, as for *A. thaliana*.

These similarities in tissue specificity between rice and Arabidopsis indicates that the
20 rice orthologue provided herein as SEQ ID NO: 3 is a true orthologue of the *A. thaliana* *ERECTA* allele set forth in SEQ ID NO: 1, with similar function

EXAMPLE 10

- 25 Demonstration of a functional role for the rice *ERECTA* gene
in modulating transpiration efficiency

To determine a functional role for the rice *ERECTA* gene (SEQ ID NO: 3), lines of rice plants carrying transposon insertions that affect expression of that gene are analyzed.

30

Nine such mutants were identified in the publicly available collection of transposon TOS17 insertional mutants at the Japanese NIAS Institute. The TOS17 retrotransposon is described in detail by Hirochika, *Current Opinion in Plant Biology*, 4, 118-122, 2001 and by Hirochika *Plant Mol Biol* 35, 231-240, 1997, which is incorporated herein by
5 reference. The nine mutant lines were identified through the website URL <http://tos.nias.affrc.go.jp/~miyao/pub/tos17/>, and they have the accession numbers NG0578 (mutant A), ND3052 (mutant B), ND4028 (mutant C), NC0661 (mutant D), NE1049 (mutant E), NF8517 (mutant F), NE8025 (mutant G), NE3033 (mutant H) and NF8002 (mutant I).

10

Nine transposon insertional mutants were ordered from NIAS, that carry the TOS17 stable retrotransposon insert in various parts of the *ERECTA* gene in the Nipponbare background, the genotype used for rice genome sequencing: NG0578, ND3052, ND4028, NC0661, NE1049, NF8517, NE8025, NE3033 and NF8002.

15

The transposon insertions in these nine lines affect the membrane spanning region of the protein (mutants I, D, E) or the Leucine Rich Repeat (LRR) domains (mutant H and G) in LRR 7 and LRR 18, respectively. In mutant B, TOS17 alters the coding sequence just upstream of sequences encoding the protein kinase domain I. In mutants C and F,
20 the TOS17 insertion alters the sequence encoding domain VIa of the *ERECTA* protein. In mutant A, the TOS7 insertion is in a sequence encoding a region between domains IX and X. The sequence information on these mutants is publicly available from the NIAS website.

25 Using mutant seed for lines A-I received from NIAS, plants were grown for amplification of seed and analysis. Except in two mutants where several plants died, plants look healthy, with good growth indicating that, as in *Arabidopsis*, there is great potential to alter the *ERECTA* gene towards altered transpiration efficiency without adversely affecting growth and/or yield.

30

Based upon sequence information for each mutant A-I, primers were designed to amplify the mutant *erecta* alleles from seedling material derived from 20 seeds. Amplification is performed under standard conditions, to identify for each mutant, plants that are homozygous, heterozygous or null at the *ERECTA* locus. Homozygous
5 TOS17 mutants B and E, and heterozygous lines and null lines in all lines A-I were identified.

In parallel to gaining information on whether or not the mutant lines were homozygous or heterozygous or null mutants, specific plant parts are removed for analysis of the
10 consequences of the mutations on the *ERECTA* gene expression (levels and tissue specificity of expression), using quantitative real-time PCR as described herein. Additionally, the transpiration efficiency phenotype of each mutant line is determined by measuring C&O isotopic composition and ash contents of plant samples.

15 Initial results on ^{13}C isotopic composition of mature blades of rice seedlings reveals significant variation between mutant lines (-32.8 to -34.2 per mil) and, in at least 4 mutants, significant deviations from the wild type values, towards more negative values, suggesting that the *erecta* mutations do affect transpiration efficiency in rice, as in Arabidopsis.

20

Similar methods as above are applied to analyzing the progeny of the mutant plants, to facilitate analysis of the effects of the *erecta* mutations under a range of conditions, including flooding (as is the most common practice for Nipponbare), water stress such as from soil drying (upland rice growth conditions) or low air humidity (heat spells).
25 Differences in plant morphology, anatomy and apical dominance are noted under each environmental condition. Parameters that are characterised include tillering patterns, the anatomy of leaves and meristems, development and growth rates.

Comparisons between mutants A-I are further used to characterize the role of the
30 different protein domains in conferring different phenotypes observed for each line under different environmental and/or agricultural growth conditions. It is interesting

that, among the 4 mutants that exhibit much lower C isotopic composition than the wild type, three are those mutants where the TOS17 insert affects the membrane spanning region.

5

EXAMPLE 11

Effect of silencing *ERECTA* gene expression on transpiration efficiency

To confirm the role of the *ERECTA* gene in conferring the transpiration efficiency phenotype on a plant, expression of the wild-type *ERECTA* allele is reduced or inhibited using standard procedures in plant molecular biology, such as, for example, antisense inhibition of *ERECTA* expression, or the expression of inhibitory interfering RNA (RNAi) that targets *ERECTA* expression at the RNA level. All such procedures will be readily carried out by the skilled artisan using the disclosed nucleotide sequences of the *ERECTA* genes provided herein or sequences complementary thereto.

For transformation of rice and *Arabidopsis*, transgenes are prepared in disarmed, non-tumorigenic binary vectors carrying T-DNA left and right borders and a selectable marker operable in *E Coli*.

20

Binary vectors used for DNA transfer include vectors selected from the group consisting of:

1. pPZP222 (Hajdukiewicz et al, 1994, *Plant Mol Biol* 25, 989-994);
2. PBI 121 (Clontech) (Ueda et al 1999, *Protoplasma* 206, 201-206);
- 25 3. pOCA18 (Olszewski et al 1988, *Nucl. Acid Res*, 16 10765-10782);
4. pGreen and pSoup or variants thereof (Hellens et al., 2000, *Plant Mol Biol* 42, 819-832) and
5. binary vectors developed on the pCAMBIA vectors backbone described at the webiste of CAMBIA.

30

The starting material for all these vectors was the backbone developed by Hajdukiewicz et al., 1994. The pPZP series of vectors comprise (i) a wide-host-range origin of replication from the *Pseudomonas* plasmid pVS1, which is stable in the absence of selection; (ii) the pBR322 origin of replication (pMB9-type) to allow high-yielding DNA preparations in *E. coli*; (iii) T-DNA left (LB) and right (RB) borders, including overdrive; and (iv) a CaMV35S promoter expression cassette. While the pPZP series of vectors also served as the backbones for the pCAMBIA series, they have been very extensively modified for particular applications.

- 10 Vectors containing in their T-DNA various combinations of the following components are particularly preferred:
 1. hptII resistance gene cassette for conferring resistance to hygromycin on transformed plant material, wherein expression of hptII is operably under control of Ubi1 or 35S promoter;
 - 15 2. a reporter gene cassette comprising nucleic acid encoding the EGFP (Enhanced Green Fluorescence Protein) and/or beta-glucuronidase (GUS and GUSPlus) reporters;
 3. Gal4/VP16 transactivator cassette; and
 4. one or more plant gene expression cassettes comprising either full-length or partial cDNAs of *ERECTA* genes in the sense or antisense orientation, or capable of expressing RNAi comprising sequences derived from the *ERECTA* gene, including any genomic fragments of plant DNA.

The binary vectors are transferred to disarmed strain AGL1 of *Agrobacterium tumefaciens* by standard tri-parental matings (Ditta et al, 1980, *Proc. Natl Acad. Sci.* 77,7347-7351) using the pRK2013 helper strain of *E. coli*. *A. thaliana* plants are transformed using the standard floral dip method for transformation by disarmed strains of *A. tumefaciens* (Clough and Bent, 1998, *The Plant Journal* 16, 735-743). Rice is transformed by generating embryogenic calli from excised embryos and subjecting the embryogenic calli to *Agrobacterium tumefaciens* mediated transformation according to published procedures (eg Wang et al 1997, *J Gen and Breed*, 51 325-334, 1997).

Transformed plants are analyzed to confirm that those lines expressing antisense or RNAi constructs have reduced expression of functional ERECTA protein and more closely resemble the *erecta* phenotype than do wild-type plants or plants ectopically
5 expressing a wild-type *ERECTA* gene in the sense orientation.

EXAMPLE 12

Identification of a sorghum ortholog of *A. thaliana ERECTA*

10

An ortholog of the *A. thaliana ERECTA* allele (SEQ ID NO: 1) in sorghum was identified *in silico* by homology searching of the NCBI protein database using the BLAST programme under standard conditions. The input sequence was SEQ ID NO: 2. The nucleotide sequence of the sorghum ortholog is presented in SEQ ID NO: 3,
15 with the encoded protein comprising the amino acid sequence set forth in SEQ ID NO: 4.
6.

EXAMPLE 13

20

Identification of *A. thaliana ERECTA* homologs

Two homologs of the *A. thaliana ERECTA* allele (SEQ ID NO: 1) were identified *in silico* by homology searching of the NCBI protein database using the BLAST programme under standard conditions. The input sequence was SEQ ID NO: 2. The
25 nucleotide sequences of the *A. thaliana ERECTA* homologs are presented in SEQ ID NOs: 7 and 9, with the encoded proteins comprising the amino acid sequences set forth in SEQ ID NOs: 8 and 10, respectively.

T-DNAinsertional mutants for these two homologous genes, both on chr5, have been
30 identified in the Salk Institute mutant collection (web address: signal.salk.edu/cg-bin/tdnaexpress). Several of these mutants were ordered: Salk_007643 and

Salk_026292 for gene At5g07180; Salk_045045 and Salk_081669 for gene At5g62230. Primer pairs were designed in order to determine insert copy number and homozygosity/heterozygosity in the seedlings grown from the seeds that were received. Homozygous lines with 1 insert were identified and are under characterisation in order
5 to compare the expression patterns (tissue localisation and mRNA levels) of the two genes and of the ERECTA gene across a range of environmental conditions and determine whether the three genes are functionally related.

EXAMPLE 14

10 Identification of wheat orthologs of *A. thaliana* ERECTA

Partial cDNA sequence of orthologs of the *A. thaliana* ERECTA allele (SEQ ID NO: 1) in wheat were initially identified *in silico* by homology searching of the NCBI protein database using the BLAST programme under standard conditions. It was necessary,
15 however, to conduct additional searches of private databases in order to link the partial sequences identified in the NCBI database. Correction of partial sequences located in the NCBI database was also necessary in order to generate a contig corresponding to the wheat ERECTA ortholog.

20 The input sequence is the *A. thaliana* (SEQ ID NO: 2) or rice (SEQ ID NO: 4) amino acid sequences or a nucleotide sequence encoding same. The nucleotide sequences of the wheat ortholog are presented in SEQ ID NOs: 11-19, with the encoded proteins comprising the amino acid sequences set forth in SEQ ID NO: 20.

25 The sequence set forth in SEQ ID NOs: 11 to 18 are partial cDNA sequences. The corresponding sequence of the wheat ERECTA ortholog (SEQ ID NO: 19) is isolated by standard nucleic acid hybridization screening of a wheat cDNA library.

To confirm the role of the wheat ERECTA orthologs in transpiration efficiency,
30 expression data sets are used for *in silico* studies of ERECTA gene expression in a range of tissues of wheat plants grown under a range of environmental conditions,

thereby providing indications of tissue specificities in expression patterns and preliminary data on the types of environments where the *ERECTA* ortholog is most likely to play a physiological role in relation to water use in this species. In these studies, nucleic acid comprising the sequence set forth in SEQ ID NO: 11 to 19, or a
5 sequence complementary thereto, are used to produce hybridization probes and/or amplification primers.

Additionally, an *ERECTA* gene (SEQ ID NO: 11 to 19) in the sense or antisense orientation is introduced into wheat, thereby producing transformed expression lines.
10 Gene constructs are specifically to silence *ERECTA* gene expression using RNAi technology, or alternatively, to ectopically express the entire open reading frame of the gene.

Based upon similar function, the open reading frame of the *A. thaliana ERECTA* gene
15 (i.e., SEQ ID NO: 1) is also introduced into wheat plant material in the sense orientation, thereby ectopically expressing *A. thaliana ERECTA* in wheat.

Gene constructs are introduced into wheat following any one of a number of standard procedures, such as, for example, using *A. tumefaciens* mediated transformation as
20 described in published AU 738153 or EP 856,060-A1 or CA 2,230,216 to Monsanto Company, or using published biolistic transformation methods as described by Pellegrineschi *et al.*, *Genome* 45(2), 421-30, 2002. Accordingly, genetic transformation is readily used to generate wheat lines with altered expression of an *ERECTA* gene. About 30 to 40 different transformants are produced, depending upon
25 the efficiency of RNAi in reducing expression of *ERECTA* in wheat.

Primary transformants (T0) are characterized to determine the number and loci at which transgenes are inserted. T1 and T2 segregating progenies are then generated from selected T0 transformants, and analyzed to determine segregation ratio and to
30 confirm the number of loci having inserted transgenes. Those T1 and/or T2 lines

having single transgene insertions are selected and used to generate and multiply seed for physiological studies.

Water use efficiency in the T1 and/or T2 lines is determined through (a) gravimetric
5 measurements of water transpired and biomass increases; (b) ^{13}C isotopic discrimination in plant tissues, (i.e., by determining Δ ; and (c) ash content of plant tissue.

Meristem and leaf development are also analyzed, especially with respect to the
10 differentiation and anatomy of the epidermis, the stomatal complexes and the mesophyll tissue and by examining leaf gas exchange properties. This is done using microscopy, in situ imaging techniques and concurrent on-line measurements of C isotopic discrimination (Δ) and of CO_2 and water fluxes in and out of leaves. Information on gene regulation and the network of genes in which the *ERECTA*
15 ortholog operates in its effects on transpiration efficiency, is determined by transcriptome analysis of a restricted set of the transgenic lines with altered *ERECTA* expression.

As described herein for *A. thaliana* and rice, correlations between physiological
20 measurements and gene expression level or copy number confirm the role of the ortholog in conferring the transpiration efficiency phenotype in wheat.

EXAMPLE 15

25 Identification of a maize ortholog of *A. thaliana ERECTA*

Partial cDNA sequence of ortholog of the *A. thaliana ERECTA* allele (SEQ ID NO: 1) in maize were initially identified *in silico* by homology searching of the NCBI protein database using the BLAST programme under standard conditions. It was necessary,
30 however, to conduct additional searches of private databases in order to link the partial sequences identified in the NCBI database. Correction of partial sequences located in

the NCBI database was also necessary in order to generate a contig corresponding to the maize *ERECTA* ortholog.

The input sequence was SEQ ID NO: 2. The nucleotide sequence of a maize ortholog
5 is presented in SEQ ID NOs: 21 to 44, with the encoded protein comprising the amino acid sequence set forth in SEQ ID NO: 45.

The sequence set forth in SEQ ID NOs: 21 to 43 are partial cDNA sequences. The corresponding sequence of the maize ortholog (SEQ ID NO: 44) is isolated by standard
10 nucleic acid hybridization screening of a wheat cDNA library.

To confirm the role of the maize *ERECTA* ortholog in transpiration efficiency, expression data sets are used for *in silico* studies of *ERECTA* gene expression in a range of tissues of maize plants grown under a range of environmental conditions,
15 thereby providing indications of tissue specificities in expression patterns and preliminary data on the types of environments where the *ERECTA* ortholog is most likely to play a physiological role in relation to water use in this species. In these studies, nucleic acid comprising the sequence set forth in SEQ ID NO: 15, or a sequence complementary thereto, is used to produce hybridization probes and/or
20 amplification primers.

Additionally, collections of transposon-tagged maize mutants are searched to select those having insertions that affect expression of the *ERECTA* gene and the expression level and/or copy number of the *ERECTA* ortholog is correlated to transpiration
25 efficiency under the range of environmental growth conditions, essentially as described herein for *A. thaliana* and rice.

Additionally, an *ERECTA* gene in the sense or antisense orientation is introduced into maize, thereby producing transformed expression lines. Gene constructs are specifically
30 to silence *ERECTA* gene expression using RNAi technology, or alternatively, to ectopically express the entire open reading frame of the gene.

Based upon similar function, the open reading frame of the *A. thaliana ERECTA* gene (i.e., SEQ ID NO: 1) is also introduced into maize plant material in the sense orientation, thereby ectopically expressing *A. thaliana ERECTA* in maize.

5

Gene constructs are introduced into maize following any one of a number of standard procedures, such as, for example, any of the methods described by Gordon-Kamm et al., *Plant Cell* 2(7), 603-618, 1990; US Patent No. 5,177,010 to University of Toledo; US Patent No. 5,981,840 to Pioneer Hi-Bred; or published US application No. 10 20020002711 A1 (Goldman and Graves);. Accordingly, genetic transformation is used to generate maize lines with altered expression of an *ERECTA* gene.

About 30 to 40 different transformants are produced, depending upon the efficiency of RNAi in reducing expression of *ERECTA*.

15

Primary transformants (T0) are characterized to determine the number and loci at which transgenes are inserted. T1 and T2 segregating progenies are then generated from selected T0 transformants, and analyzed to determine segregation ratio and to confirm of number of loci having inserted transgenes. Those T1 and/or T2 lines having 20 single transgene insertions are selected and used to generate and multiply seed for physiological studies.

Water use efficiency in the T1 and/or T2 lines is determined through (a) gravimetric measurements of water transpired and biomass increases; (b) ¹³C isotopic 25 discrimination in plant tissues, (i.e., by determining Δ); and (c) ash content of plant tissue.

Meristem and leaf development are also analyzed, especially with respect to the differentiation and anatomy of the epidermis, the stomatal complexes and the 30 mesophyll tissue and by examining leaf gas exchange properties. This is done using microscopy, in situ imaging techniques and concurrent on-line measurements of Δ and

of CO₂ and water fluxes in and out of leaves. Information on gene regulation and the network of genes in which the *ERECTA* ortholog operates in its effects on transpiration efficiency, is determined by transcriptome analysis of a restricted set of the transgenic lines with altered *ERECTA* expression.

5

As described herein for *A. thaliana* and rice, correlations between physiological measurements and gene expression level or copy number confirm the role of the ortholog in conferring the transpiration efficiency phenotype in maize.

10

EXAMPLE 16

Mechanism of enhanced transpiration efficiency and inheritance of *ERECTA* in Arabidopsis (Landsberg and Columbia backgrounds)

15 The present inventors performed direct measurements of transpiration efficiency (ratio of CO₂ assimilation rate to transpiration rate) in both Landsberg and Columbia backgrounds. To confirm the role of *ERECTA* under a wider range of agronomically relevant conditions, the transpiration efficiencies of transformed plants carrying an *ERECTA* allele in response to varying environmental conditions (i.e., soil water and ion
20 content, atmospheric humidity and CO₂ levels) were determined and compared to the response of wild type plants (e.g., ER). Results of these experiments are presented in Figures 6-11, and Tables 8 and 9.

Data in Figures 6 show that the enhanced transpiration efficiency obtained by inserting
25 a transgene carrying the wild type ER allele in the Ld-er1 mutant (line T2+ER) is mostly due to a decreased stomatal conductance. The phenotype of the transgenic line (T2+ER in graphs) is similar to that of a Ld-ER ecotype near isogenic to Ld-er1 obtained from the Stock Centre (line 3177 on graphs). The increased transpiration efficiency in transgenic ER, compared to levels observed in wild type ER line is
30 observed under both current ambient CO₂ levels and increased CO₂ levels that are within the limits predicted to occur worldwide over the next two decades.

Data in Figure 8 show that the reduced stomatal conductance in the ER T2 transgenic line compared to the Ld-er1 line is, at least for a large part, caused by a reduced stomatal density (decrease in the number of stomata per unit area by more than half,
5 down to similar levels as those observed in wild type Ld-ER). This decrease in stomatal density is relatively higher than that in the density of epidermal cells whose surface area is increased by only about 10%. It therefore follows that the ER transgene has affected stomatal development, specifically, and caused a decreased in stomatal index. These data show complementation with respect to the processes driving variation in
10 transpiration efficiency.

Reciprocal crosses were also performed between the two parental lines NW20 (Ld-er1) and Col4 (Col-ER). The notation F1 (Col*Ld) refers to the F1 plants where Col was the recipient of Ld pollen, while the notation F1 (Ld*Col) indicates the converse (Ld ovary
15 receiving Col pollen). Initial analysis of these two types of F1 plants has been made for: gas exchange and photosynthetic properties, transpiration efficiency (Figure 7) and C isotopic composition (Table 9), rosette shape and developmental rate, anatomy of leaf epidermis (Figure 8), flowering date, inflorescence and pod shape. Consistent with our analysis of complementation experiments, the data show that the *ERECTA* gene
20 affects all these phenotypes and not only inflorescence and pod shape.

The data also show a complex inheritance of the *ERECTA* gene, such that the gene is dominant, with no reciprocal effect on pod shape (longer pods, longer stems and pedicels in all F₁ plants, similar to the Col -ER parent). However, for other traits,
25 results indicate maternal effects: hence the transpiration efficiency values (see Figure 7a) and rosettes carbon isotope composition in F₁ plants (Table 9) are intermediate between the parental values, but different between the two sets of F₁ plants: values for F₁ plants (Col*Ld) are closer to the Col values, while those for F₁ plants (Ld*Col) are closer to values for the Ld parent.

Data in Figure 8 indicate that stomatal conductance (transpiration per unit leaf area, Figure 8a) displays values close to the Ld-er1 parent in all F1 plants, despite the stomatal densities being close to the Col-ER parent (Figure 8c). This shows that the ER gene affects not only epidermis development but also stomatal aperture (dynamics of stomata) and that while the ER effect on stomatal density appears to be dominant, effect on stomatal aperture is not.

Data in Figure 9 show the effect of various er mutations (in Col background, mutants obtained from the Stock Centre or Dr Torii) on the number of stomata per unit leaf area. The stomatal densities for all but two of those mutants are greater than those the ColER wild type leaves, and confirm the effect of the ERECTA gene on that parameter.

Data in Figure 10 show that enhanced transpiration efficiency in the ER transgenic line compared with null Ld-er1 (no insertion of transgene) is confirmed by the less negative C isotopic composition values measured in leaf material (compare values for lines NW20 and CS20 (Ld er1; lines 16 and 17 on x-axis) and a transgenic T2 ld-ER line, homozygous for the ER transgene (line 19 in the Figure). The C isotopic values measured in the ER transgenic line are similar to those in the near isogenic Ld-ER ecotype (line 18 in Figure 10). This demonstrates complementation on this phenotypic trait, and validates once again the use of C isotopic composition as a quantitative indicator (substitute) of transpiration efficiency.

Data in Figure 10 also the C isotopic compositions of a range of Col-er mutants, including those analysed in Figure 9 for stomatal densities. Most mutants show more negative C isotopic values than the COL-ER ecotype. This is consistent with the increased stomatal densities described in Figure 9 and with all other comparisons of C isotopic compositions or direct measurements of transpiration efficiencies in er/ER lines and again indicative of the positive effect of the ER allele on transpiration efficiency.

A few mutants in Figure 10 stand out, eg Col-er105, or line 3140 (a line from NASC carrying the *er1* and *gll-1* mutations). As genetic information is available for these mutants (nature and position of mutations) these mutants provide very useful functional information on the protein domain(s) of the *ERECTA* protein that are essential for
5 conferring the transpiration efficiency phenotype and underlying processes.

The present inventors also perform direct measurements of transpiration efficiency (ratio of CO₂ assimilation rate to transpiration rate) in several T2 transformants generated in a Columbia background (i.e. transformation of mutant *er-105* and *er-2/106*
10 above). Results from these measurements are shown in Figure 11. These data show that the phenotype can be complemented in a Columbia background, as determined by measuring transpiration efficiency, transpiration and CO₂ assimilation rates. Complementation is observed under conditions of both high humidity and low humidity, hence the demonstration that the *ERECTA* gene plays a role in the control of
15 transpiration efficiency under both well watered and drought conditions, and that overexpression of that gene has the potential of increasing growth and resistance to drought and drought related stresses.

More particularly, the data in Figure 11 demonstrate the role of the *ERECTA* gene on
20 transpiration efficiency across a range of humidities, including low humidities such as prevail in warm and dry areas:

- the *er-105* mutant which carries a knock-out mutation of *ERECTA* (quasi no *ER* transcript) (open black squares) in Col0 background has lower transpiration efficiency than the wild type near isogenic Col0 (open triangles).
- 25 - this mutant was transformed with an *ER* transgene under the 35S promoter and several *ER* homozygous T2 lines were produced (solid circles). Those lines (5 independent transformants are included in the graph) have much increased transpiration efficiencies (+40 to 70%) compared to the null lines (solid squares) and similar to those measured for the wild type *ER-Col0* line, across
30 the whole range of leaf to air vapour pressure deficit tested In our experiments.

Additionally, null lines that carry no transgene insertion but went through transformation and selection on antibiotics display similar values as the starting er-105 mutant demonstrating that these manipulations themselves have no detectable confounding effect on transpiration efficiency.

TABLE 1
QTL Analysis of Carbon Isotope Discrimination in Lister and Dean's Recombinant Inbred Lines

RUN No. Experimental conditions	chr2 locus (cM)	QTL analysis method	chr4 locus (cM)	CONCLUSION	
				QTLs number	predicted map position
Run 1 (40 lines) Glasshouse- 12h day length					
irradiance 150-350 $\mu\text{E m}^{-2} \text{s}^{-1}$	58.5	SIM&CIM		2	chr2: 58.5-61.02
Seedlings transferred from agar plates	46.77	SIM&CIM			chr2: 46.77-50.75
	61.02	SIM	108.5		
Run1 data but with using different markers					
	56.94 to 58.00	CIM&SIM		1	
	46.77 to 50.75	SIM			
	63.02				

RUN No. Experimental conditions	chr2 locus (cM)	QTL analysis method	chr4 locus (cM)	CONCLUSION		
				QTLs number	predicted map position	
Run 1 with different number of lines						
	58.5 to 61.02			2		
	56-61					
Run2 Glasshouse September from seeds sown on soil						
	batch 1	CIM (QTL cart)		2	chr2:	56.94-61.02
		MQTL			chr2:	50.75
	batch 2	MQTL		NS		
	batch3-5					
all batches	58.5	MQTLcart		NS		
	56.94-58.5	MQTL				

RUN No.	Experimental conditions	chr2 locus (cM)	QTL analysis method	chr4 locus (cM)	CONCLUSION	
					QTLs number	predicted map position
Run 3	37 lines: parents and lines with crossing-overs on chromosome 2 5 growth conditions differing in humidity, irradiance, mode of establishment (seeds sown on soil or seedlings transplanted from agar)					
batch B		61.02-61.06		108 NS		
batch C		56.94-58.00				
batch D		63.02	QTLcar			
		63.02	MQTL			
all batches (conditions)		58.5			1 or 2	chr2: 56.94-58.5
		61.02			3	chr2: 61.02-63.02

RUN No. Experimental conditions	chr2 locus (cM)	QTL analysis method	chr4 locus (cM)	CONCLUSION		
				QTLs number	predicted map position	
Run 4 same lines as Run 3 growth chambers 10h daylight						
	50.74				chr2:	50.74
Run 5 repeat of run 1 BUT ALL lines						
	50.74			1	chr2:	50.74
Run 7 same lines as in run 1 but in growth chamber and higher light						
10h daylength	46.77-50.75	CIM&SIM		1	chr 2:	46.77-5065
470-510 $\mu\text{E m}^{-2} \text{s}^{-1}$ irradiance						

TABLE 2

Background	Mutation	Stock Centre name	Isogenic <i>ER</i> line and Stock Centre Name
Landsberg	<i>er1</i>	CS20 or NW20 ^a	3177 or CS163
Columbia	<i>er2^b/er106</i>	3401	Col1 or 3176
Columbia	<i>er105^c</i>		Col3 with gl1 marker or Col0

a, NW20 is an *Ler* parent for Lister and Dean's recombinant lines, carrying the *er1*

5 mutation. Lines 3177 or CS163 are the closest isogenic ER lines.

b, *er2* is an *er* allele identified by Rédéi in Col background. Col1 or 3176 are the closest Col near-isogenic lines. The *er2* is same mutation as mutation *er-106* later reported by Torii and collaborators (Lease et al. 2001)

c, *er105* was isolated from a fast-neutron-irradiated Col seed population (Torii *et al.*,
10 1996).

d, Col4, the *Col* parent for the Lister and Dean's parent was systematically included in all comparisons.

TABLE 3 Comparison of er/ER lines in both Col and Ld background for carbon isotope discrimination values (per mil) in leaf material under a range of environmental conditions									
Run No.	Differences in mean carbon isotope discrimination values (per mil)								
	(1) <i>er-ER</i> (all lines)	(2) er105-Col0	(3) er105-Col4	(4) er105-3176	(5) er2-3176	(6) er1-3177	(7) er1-Col4 (parental lines for RILs)	(8) er105-Coli	(9) er1-Coli
1	0.13	0.16						0.16	
2	0.89	1.18						1.18	
3	0.26	0.11						0.11	
4	1.12	1.60						1.60	
5	1.03		1.83	1.67	0.92	0.64	0.82	1.75	0.73
6	0.70	1.13	1.01	0.71	0.27	0.74	0.73	0.95	0.73
7	0.70	1.32	1.12	1.23	0.75	0.35	0.05	1.22	0.15
8	0.59	1.16	1.11	1.19	0.54	0.28	0.06	1.16	0.17
9	0.30	1.09	0.77	0.77	0.02	0.00	0.56	0.88	0.28
10	0.56	1.05	0.94	0.87	0.38	0.39	0.33	0.95	0.36

Run #	(1) <i>difference er-ER (all lines)</i>	(2) er105-Col0	(3) er105-Col4	(4) er105-3176	(5) er2-3176	(6) er1-3177	(7) er1-Col4	(8) er105-Coli	(9) er1-Coli
11	0.48		0.40				0.52	0.40	0.52
12	0.36	0.82	1.31	1.08	0.33	0.05	0.07	1.07	0.01
13	0.38		0.90	0.82	0.07	0.60	0.52	0.86	0.56
14	0.65	1.42	0.60			0.58	0.06	1.01	0.32
15	0.82						0.82		0.82
For all runs:									
Mean	0.60	1.01	1.00	1.04	0.41	0.40	0.41	0.95	0.42
S.E.	0.07	0.14	0.11	0.11	0.10	0.08	0.09	0.12	0.08
For Common runs:									
Mean:	0.58	1.10	1.12	1.04	0.41	0.38	0.39	1.11	0.37
S.E.	0.08	0.05	0.11	0.11	0.10	0.09	0.10	0.10	0.09

TABLE 4
Run 9- December 2001: Leaf gas exchange measurements in er/ER Arabidopsis lines

[illegible]

NOTE: p_a and p_i are the ambient and intercellular partial pressures of CO_2 , respectively.

TABLE 5

experimental run	LOD	P(0.05)	P(0.01)	QTL position cM gap	LOD-LOD _{0.05}	R ²	Total R ²
1	5.2861	4.7469	5.9923	39.32-50.65	0.5392	0.19	0.89
4	3.519	3.3561	4.2134	50.63-50.65	0.1629	0.29	0.57
7	9.6489	3.9627	4.9083	50.63-50.65	5.6862	0.53	0.77
9	6.1748	4.2328	5.0278	46.77-50.65	1.942	0.44	0.81
10	Zero						
	Qtl's	3.6051	4.3889				
16	11.5132	3.188	3.8896	48.96-50.65	8.3252	0.64	0.65
2(batch1)	5.5459	3.3264	4.2907	50.63-51.02	2.2195	0.26	0.40

LOD = log₁₀ of the likelihood ratio

5

TABLE 6

Summary table of the lines used for initial functional characterisation
and analysis of ER effects:

Background ecotype	Stable T2 homozygous ER transformants	Null er control
Col-er105	T8; T29; T19; T61	T18
Col-er106/er2/3401	T165; T169; T279; T290	T143
Ld-er1	T3-7K	NW20

TABLE 7

Carbon isotope composition (per mil) of 3 mature leaves, ground together
harvested 4/6/03 ie 32 days after sowing from still vegetative rosettes

	T2 ER homozygous transformants				Null transgenics (er)			background er mutant
Line:	T46	T29			T18			Col-er105
	-31.4	-31.2			-32.2			
Line:	T145	T165	T279	T290	T154	T143	T247	Col er106/er2 /3401
	-30.4	-31	-30.5	-30.8	-31.5	-32	-31.7	
<i>Average:</i>		-30.6				-31.7		-31.7
<i>se:</i>		0.15				0.12		
line	T3-7K							Ld-er1 NW20
	-30.4							-31.3

TABLE 8

Erecta alleleLine	Line name	C isotopic composition (per mil)			
		Run 14 Average	St Err	Run 18 Average	St Err
Col_{er} mutants	102K			-29.4	.011
	103K			-29.0	.10
	105C	-31.4	0.12	-30.3	.09
	105KH	-30.2		-29.3	.011
	105KS	-29.8	0.07	-29.5	.07
	3401	-30	0.21	-29.4	.05
	106C	-30.2	0.04	-29.4	.12
	108K	-30.2	0.07	-29.5	.06
	111KH	-30.4	0	-29.5	.11
	111KS	-30.2	0.11	-29.7	.12
	114K	-30.2	0.11	-29.5	.04
	116K	-29.7	0.21	-29.0	.13
	117K	-29.7	0.18	-29.3	.08
	3140			-32.2	.06
Col0_ER	1093	-29.6	0	-29.0	.10
Ld_{er}1	NW20	-30.0	0.25	-28.9	.15
	CS20	-29.9	0.08		
Ld_ER	3177	-29.4		-28.2	.09
Transgenic Ld _{er} 1 +wild type ER	3-7K	-29.5	0.07	-28.4	.10

TABLE 9

5

	C isotope composition (per mil)
Col_ER (line 933)	-28.1
F1 (Col*Ld)	-28.5
F1 (Ld*COL)	-29.3
Ld-er1 (line NW20)	-29.9

WE CLAIM:

1. A method of selecting a plant having enhanced transpiration efficiency, comprising detecting a genetic marker for transpiration efficiency which marker comprises a nucleotide sequence linked genetically to an *ERECTA* locus in the genome
5 of the plant and selecting a plant that comprises or expresses the genetic marker.
2. The method according to claim 1 wherein the genetic marker comprises an *ERECTA* allele or *erecta* allele, or a protein-encoding portion thereof
- 10 3. The method according to claim 2 wherein the genetic marker comprises a nucleotide sequence having at least about 55% overall sequence identity to at least about 20 nucleotides in length of any one of SEQ ID Nos: 1, 3, 5, 7, 9, 11 to 19 or 21 to 44 or a complementary sequence thereto.
- 15 4. The method according to claim 2 or 3 wherein the genetic marker comprises a nucleotide sequence selected from the group consisting of:
 - (a) a sequence having at least about 55% identity to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38; SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43 and SEQ ID NO: 44;
20
25
 - (b) a sequence encoding an amino acid sequence having at least about 55% identity to an amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 20 and SEQ ID NO: 45; and
30
 - (c) a sequence complementary to (a) or (b).

5. The method according to any one of claims 1 to 4 wherein the plant is selected from the group consisting of *Arabidopsis thaliana*, rice, sorghum, wheat and maize.
- 5 6. The method according to any one of claims 1 to 5 comprising linking the transpiration efficiency phenotype of the plant to the expression of the marker in the plant.
7. The method according to any one of claims 1 to 5 comprising linking a
10 structural polymorphism in DNA to a transpiration efficiency phenotype in the plant.
8. The method according to claim 7 wherein the polymorphism is determined by a process comprising detecting a restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), single strand chain polymorphism
15 (SSCP) or microsatellite analysis.
9. The method according to any one of claims 1 to 8 comprising hybridizing a probe or primer of at least about 20 nucleotides in length from any one of SEQ ID Nos: 1, 3, 5, 7, 9, 11 to 19 or 21 to 44 or a complementary sequence thereto to genomic DNA
20 from the plant, and detecting the hybridization using a detection means.
10. The method according to any one of claims 1 to 9 wherein the selected plant has enhanced transpiration efficiency compared to a near-isogenic plant that does not comprise or express the genetic marker.
- 25
11. A method of selecting a plant having enhanced transpiration efficiency, comprising:
- (a) screening mutant or near-isogenic or recombinant inbred lines of plants to segregate alleles at an *ERECTA* locus;
- 30 (b) identifying a polymorphic marker linked to said *ERECTA* locus; and
- (c) selecting a plant that comprises or expresses the marker.

12. A method of modulating the transpiration efficiency of a plant comprising introducing an isolated *ERECTA* gene or an allelic variant thereof or the protein-encoding region thereof to a plant and selecting a plant having a different transpiration
5 efficiency compared to a near-isogenic plant that does not comprise the introduced *ERECTA* gene or allelic variant or protein-encoding region.

13. The method according to claim 12 wherein the *ERECTA* gene or allelic variant or protein-encoding region comprises a nucleotide sequence selected from the group
10 consisting of:

- (c) a sequence having at least about 55% identity to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18,
15 SEQ ID NO: 19; SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38; SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43 and SEQ ID NO: 44; and
20
- (d) a sequence encoding an amino acid sequence having at least about 55% identity to an amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 20 and SEQ ID NO: 45.

25

14. The method according to claim 12 or 13 wherein the plant is selected from the group consisting of *Arabidopsis thaliana*, rice, sorghum, wheat and maize.

15. The method according to any one of claims 12 to 14 wherein the *ERECTA* gene or allelic variant or protein-encoding region is introduced to the plant by a process
30 comprising introgression.

16. The method according to any one of claims 12 to 14 wherein the *ERECTA* gene or allelic variant or protein-encoding region is introduced to the plant by a process comprising transforming plant material with a gene construct comprising the gene or
5 allelic variant or protein-encoding region thereof.
17. The method according to any one of claims 12 to 16 further comprising expressing the introduced gene or allelic variant or protein encoding region in the plant.
- 10 18. The method according to any one of claims 12 to 17 wherein transpiration efficiency is enhanced in the plant.
19. The method of claim 18 wherein the transpiration efficiency is enhanced as a consequence of the ectopic expression of an *ERECTA* allele or the protein-encoding
15 region thereof in the plant.
20. The method according to any one of claims 12 to 17 wherein transpiration efficiency is reduced in the plant.
- 20 21. The method of claim 20 wherein the transpiration efficiency is reduced as a consequence of reduced expression of an *ERECTA* allele in the plant.
22. A plant having modified transpiration efficiency compared to a near-isogenic plant wherein said plant is produced by a process comprising performing the method
25 according to any one of claims 12 to 21.
23. The plant of claim 22 selected from the group consisting of a rice plant, a wheat plant and a maize plant.
- 30 24. An isolated *ERECTA* gene from wheat comprising a nucleotide sequence selected from the group consisting of:

- (i) a sequence selected from the group consisting of: SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, and SEQ ID NO: 19;
- (ii) a sequence encoding the amino acid sequence set forth in SEQ ID NO: 20; and
- 5 (iii) a sequence that is complementary to (i) or (ii).

25. An isolated *ERECTA* gene from maize comprising a nucleotide sequence selected from the group consisting of:

- (i) a sequence selected from the group consisting of: SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43 and SEQ ID NO: 44;
- 10 (ii) a sequence encoding the amino acid sequence set forth in SEQ ID NO: 45; and
- (iii) a sequence that is complementary to (i) or (ii).

26. A gene construct comprising the isolated *ERECTA* gene according to claim 24 or 25 operably in connection with a promoter sequence that is operable in a plant.

20

27. Use of an isolated *ERECTA* gene or allelic variant or protein-encoding region thereof in the preparation of a genetic construct for modulating the transpiration efficiency of a plant.

25 28. Use according to claim 27 wherein the *ERECTA* gene or allelic variant or protein-encoding region comprises a nucleotide sequence selected from the group consisting of:

- (a) a sequence having at least about 55% identity to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18,
- 30

SEQ ID NO: 19; SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32 SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38; SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43 and SEQ ID NO: 44; and

(b) a sequence encoding an amino acid sequence having at least about 55% identity to an amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 20 and SEQ ID NO: 45.

29. A method of identifying a gene that determines the transpiration efficiency of a plant comprising:

- (a) identifying a locus associated with genetic variation in transpiration efficiency in the plant;
- (b) identifying a gene or allele that is linked to said locus, wherein said gene or allele is a candidate gene or allele for determining the transpiration efficiency of a plant; and
- (c) determining the transpiration efficiencies of a panel of plants, wherein not all members of said panel comprise or express said gene or allele, and wherein variation in transpiration efficiency between the members of said panel indicates that said gene is involved in determining transpiration efficiency.

30. A method of identifying a gene that determines the transpiration efficiency of a plant comprising:

- (a) identifying a locus associated with genetic variation in transpiration efficiency in a plant;
- (b) identifying multiple alleles of a gene that is linked to said locus, wherein said gene is a candidate gene involved for determining the transpiration efficiency of a plant; and

- (d) determining the transpiration efficiencies of a panel of plants, wherein each member of said panel comprises at least one of said multiple alleles, wherein variation in transpiration efficiency between the members of said panel indicates that said gene is involved in determining transpiration efficiency.

5

31. A method of increasing the resistance of a plant to an environmental stress comprising enhancing the level of expression of an *ERECTA* gene or allelic variant thereof or protein encoding region thereof in said plant.
- 10 32. A method of increasing seed or grain weight in a plant comprising enhancing the level of expression of an *ERECTA* gene or allelic variant thereof or protein encoding region thereof in said plant.
- 15 33. A method of increasing the number of seeds produced by a plant comprising enhancing the level of expression of an *ERECTA* gene or allelic variant thereof or protein encoding region thereof in said plant.
- 20 34. The method of any one of claims 31 to 33, the level of expression is enhanced by introducing an *ERECTA* gene or allelic variant thereof or the protein encoding region thereof to a plant.

The method of claim 34 wherein the *ERECTA* gene or allelic variant or protein-encoding region comprises a nucleotide sequence selected from the group consisting of:

- (a) a sequence having at least about 55% identity to a sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37,

SEQ ID NO: 38; SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43 and SEQ ID NO: 44; and

- (b) a sequence encoding an amino acid sequence having at least about 55% identity to an amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 20 and SEQ ID NO: 45.
- 5
35. A plant produced by the method of any one of claims 31 to 34,

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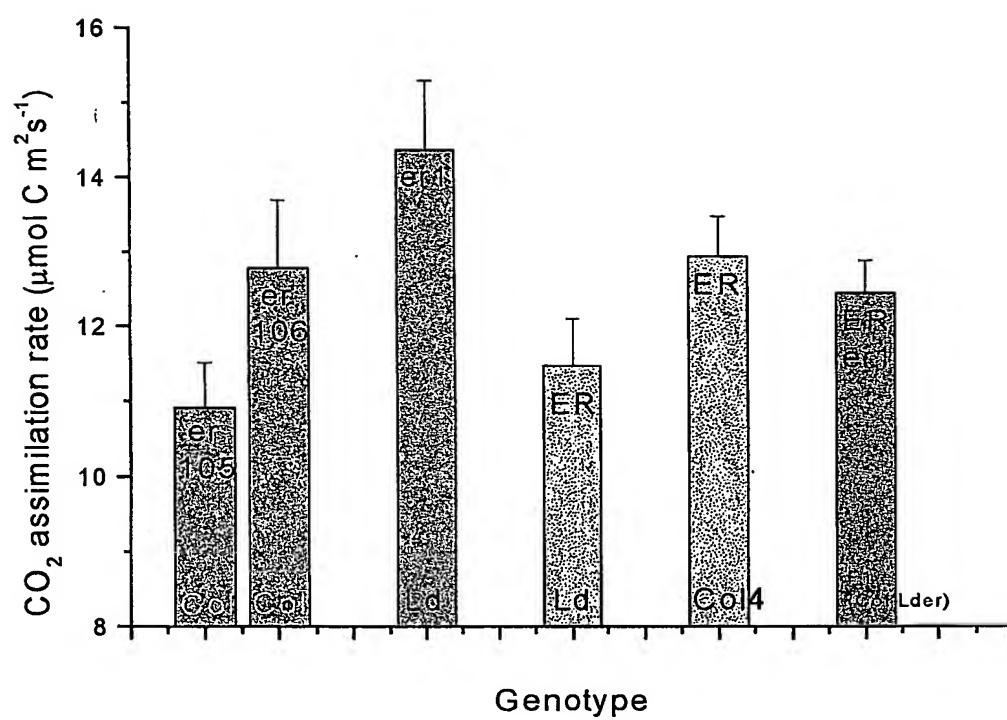


Figure 1a

2/22

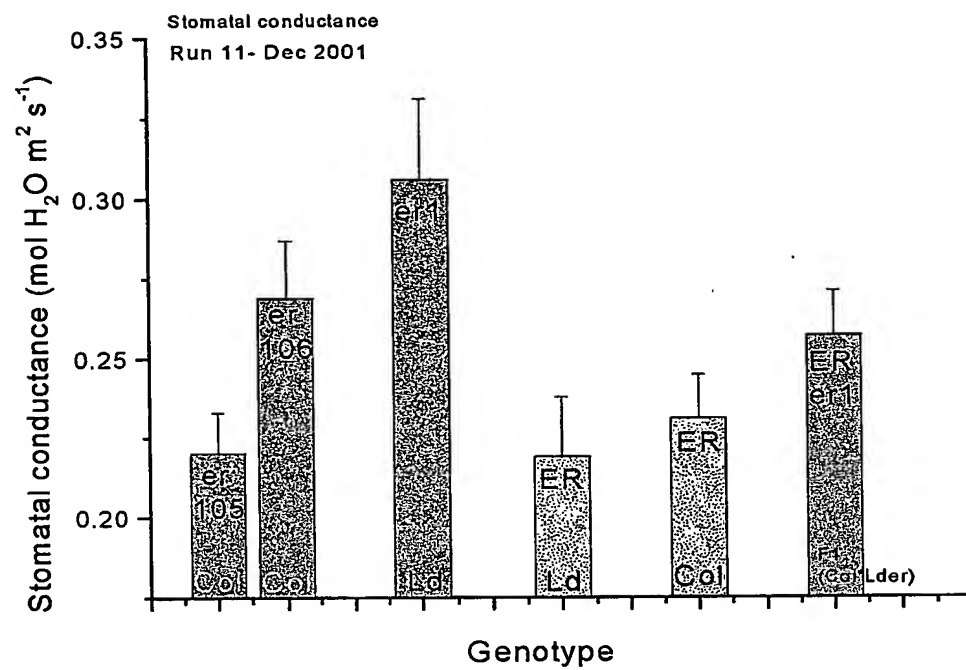


Figure 1b

3/22

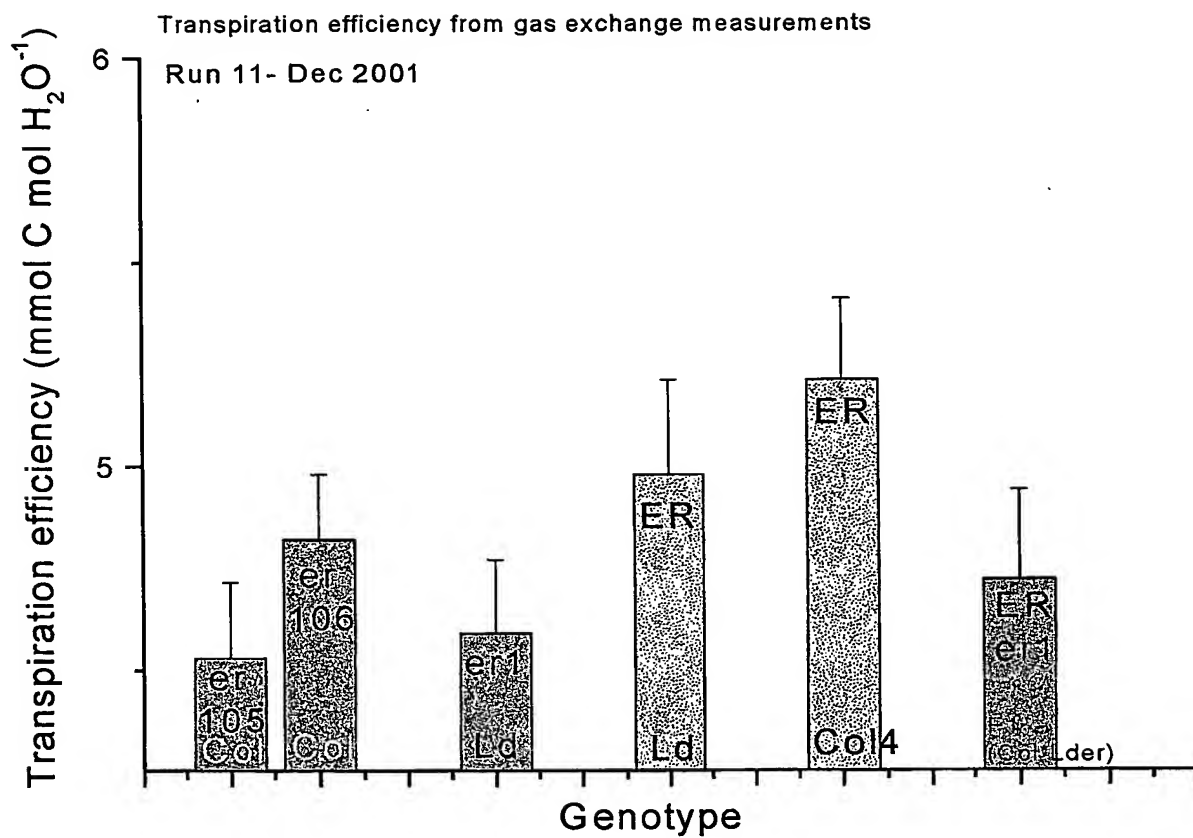


Figure 1c

4/22

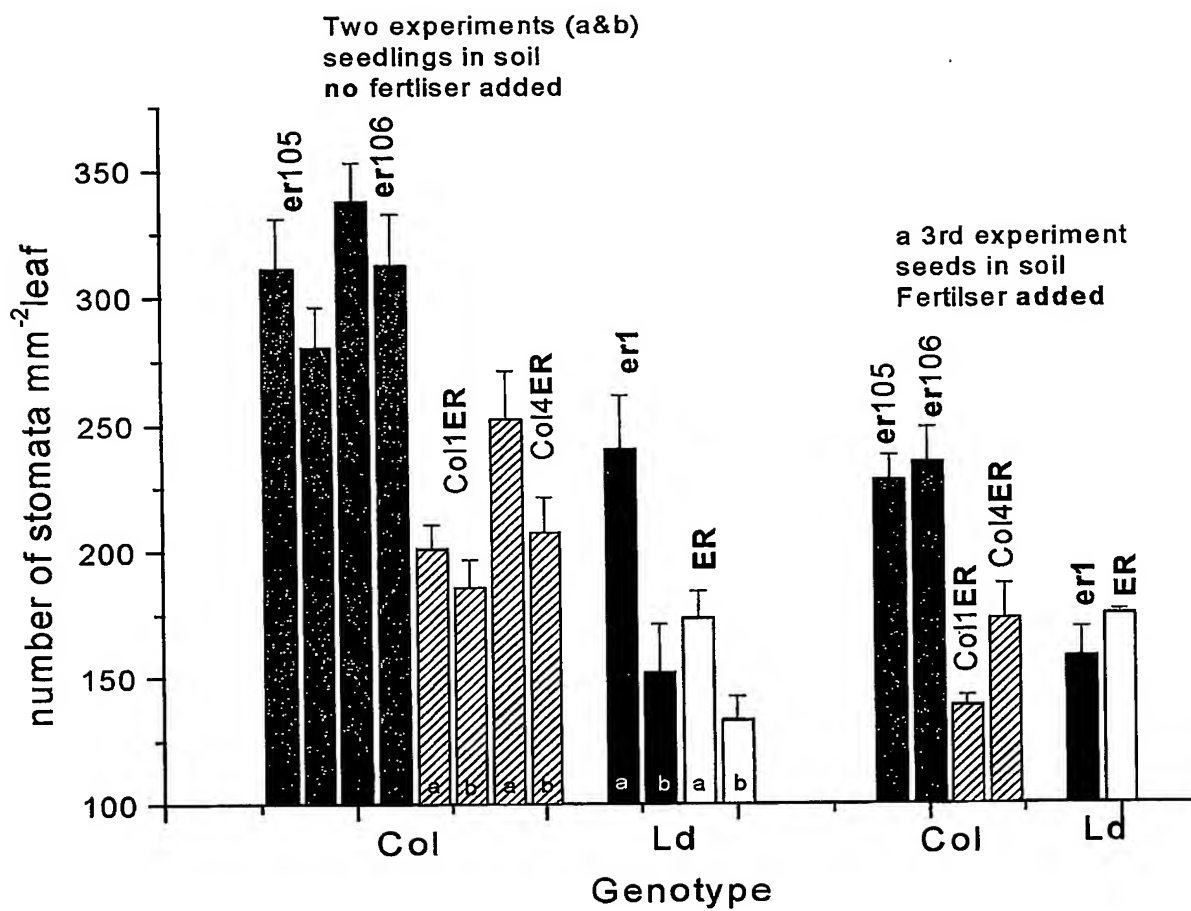


Figure 2a

5/22

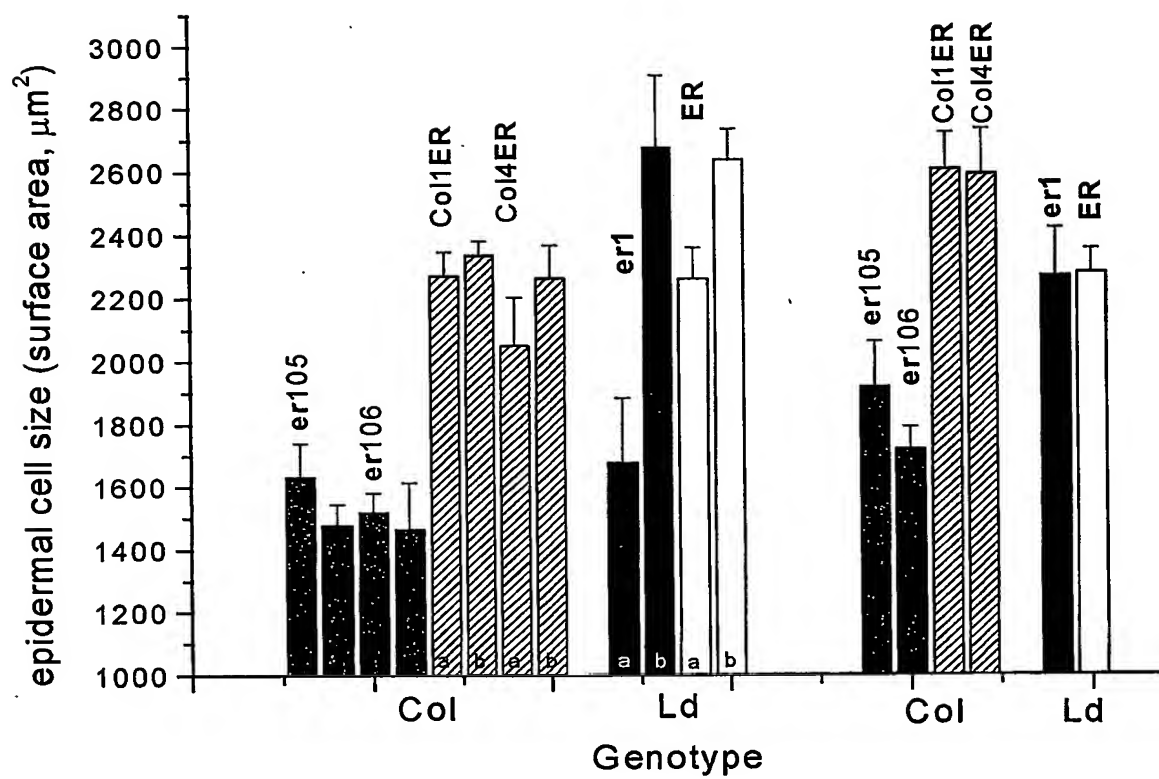


Figure 2b

6/22

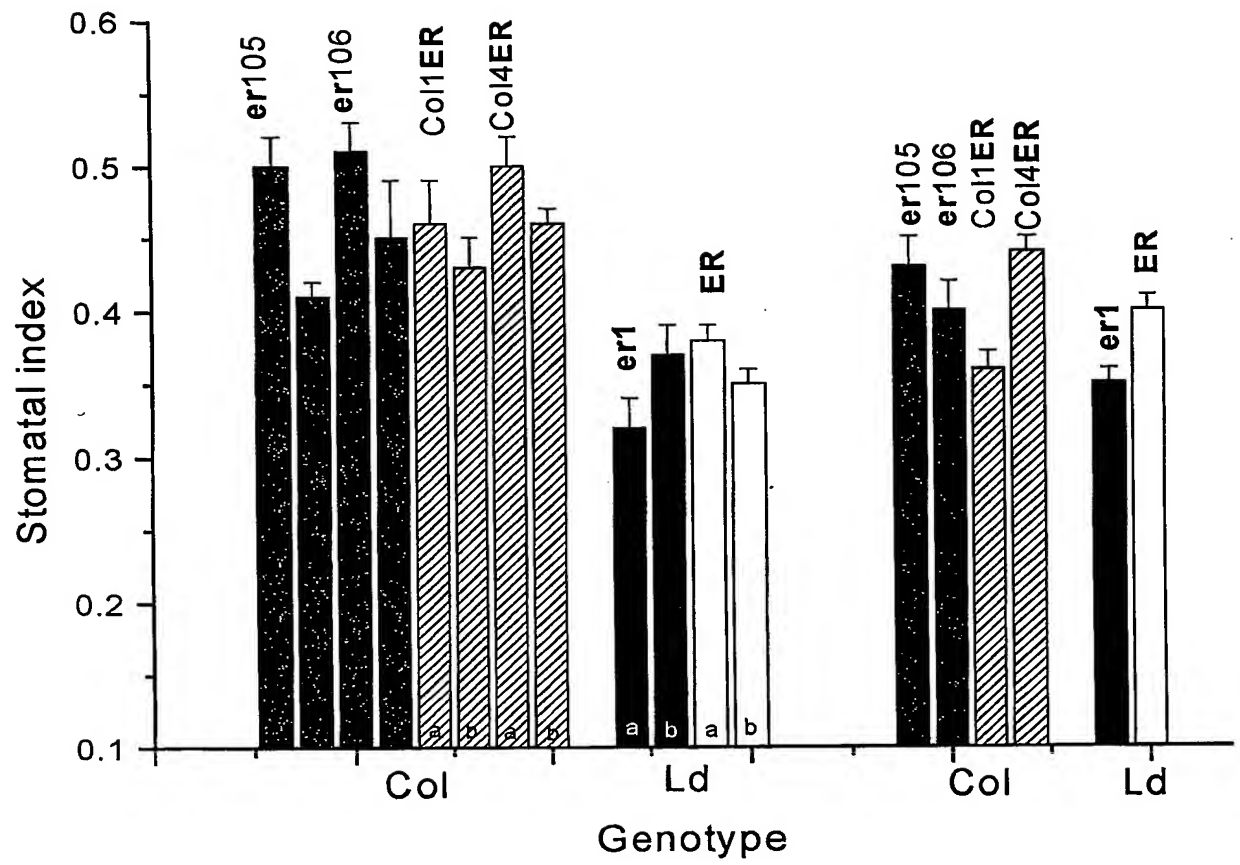


Figure 2c

7/22

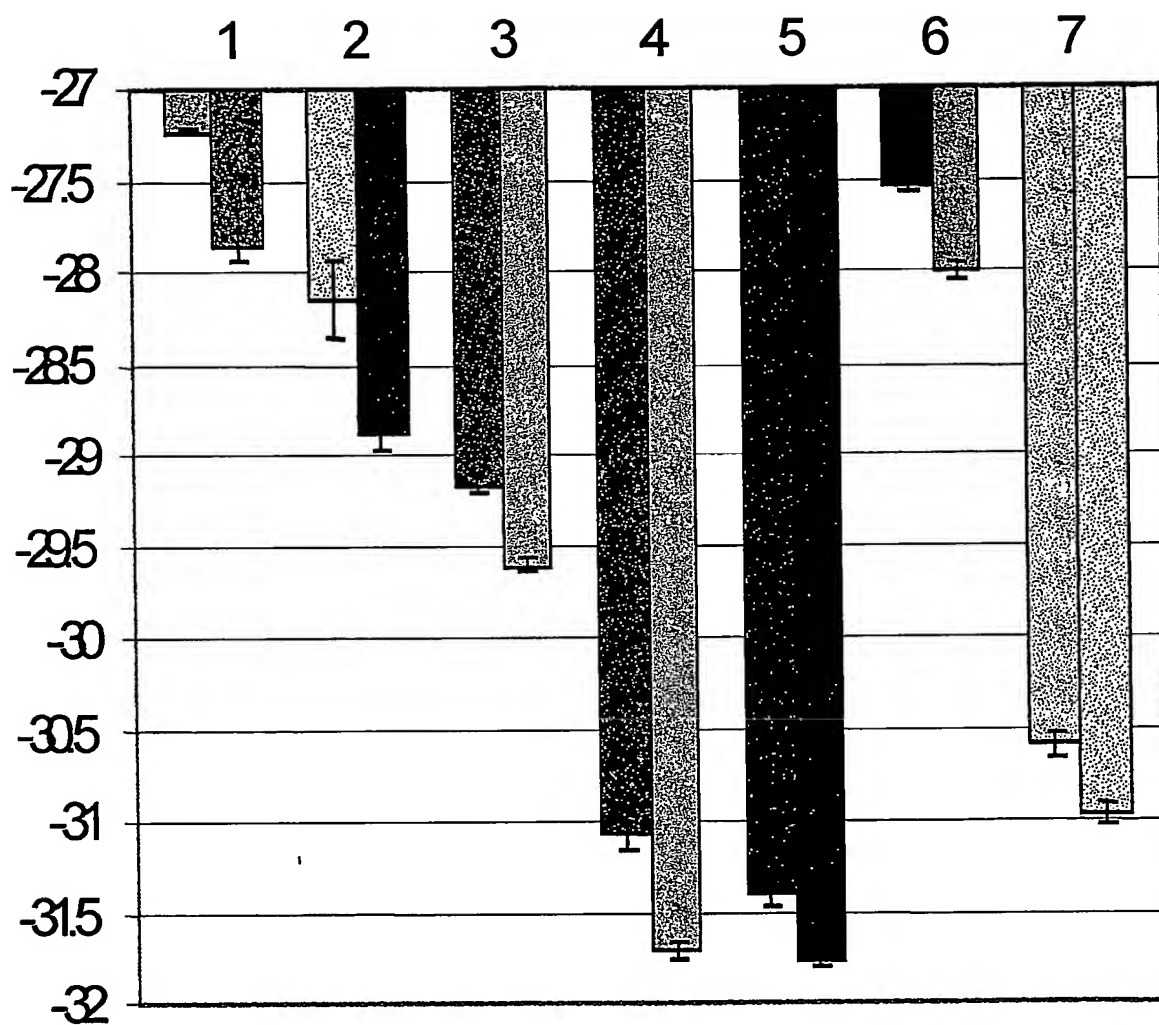


Figure 3

8/22

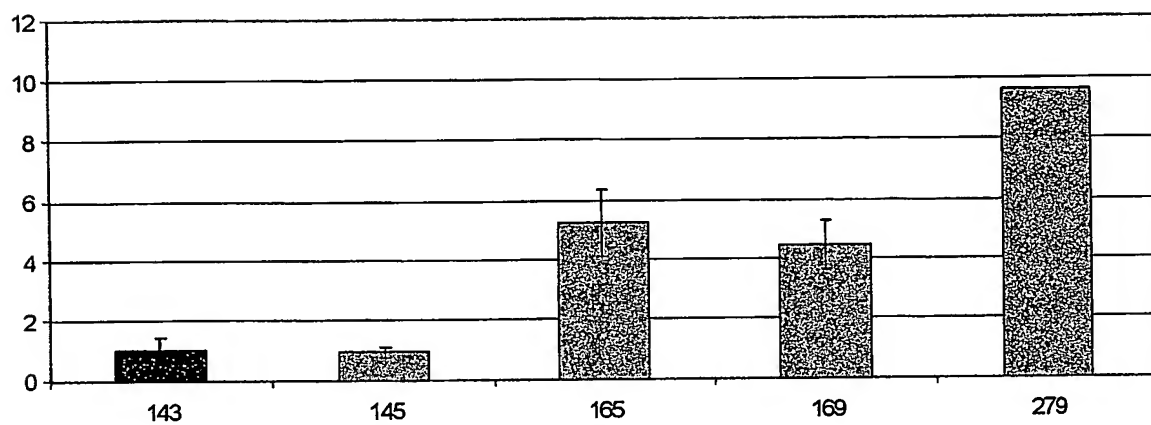


Figure 4a

9/22

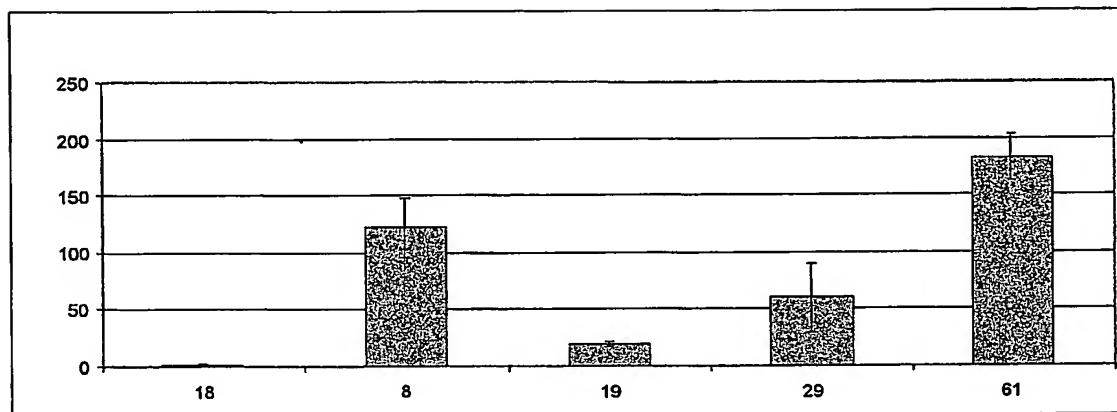


Figure 4b

10/22

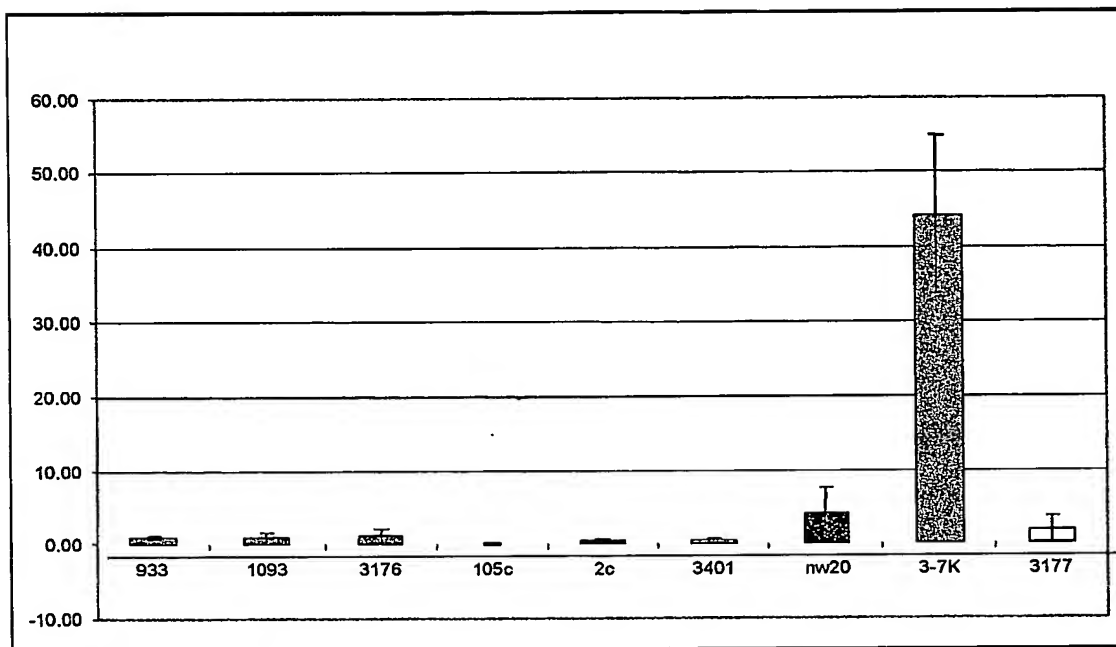


Figure 4c

11/22

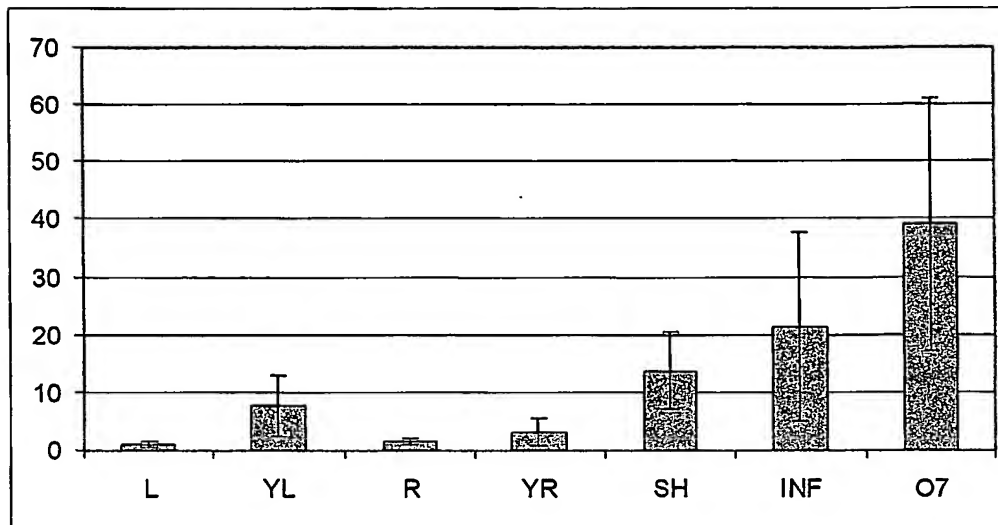


Figure 5a

12/22

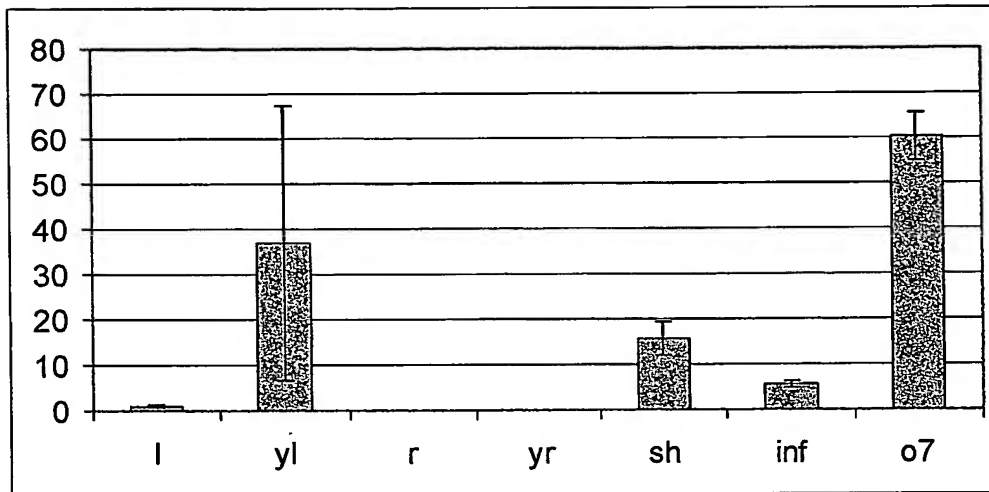


Figure 5b

Figure 6a: Transpiration efficiency at 350 and 500ppm CO₂
(mmolC mol H₂O⁻¹)

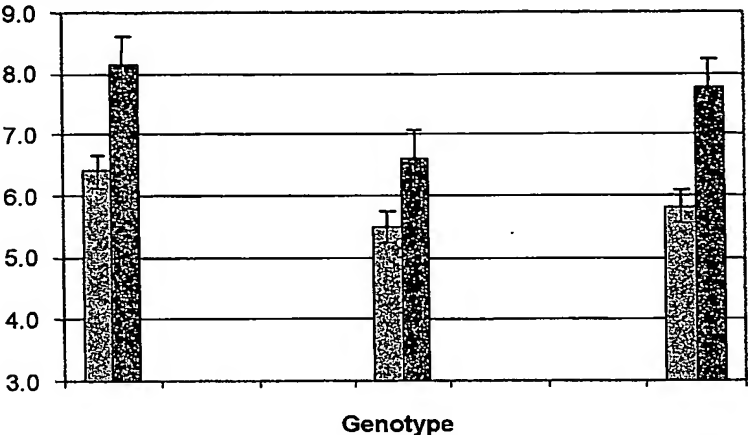


Figure 6b: CO₂ assimilation rate
(μ mol C m⁻²s⁻¹)

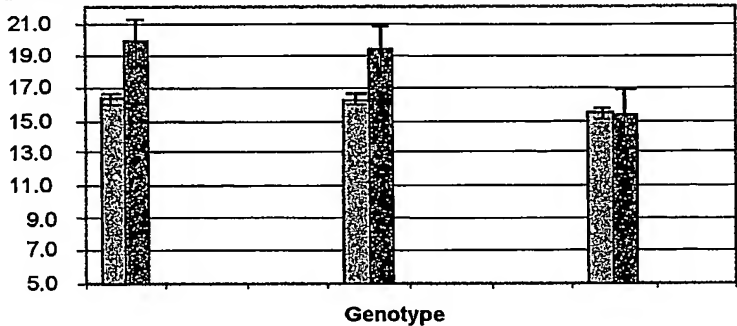
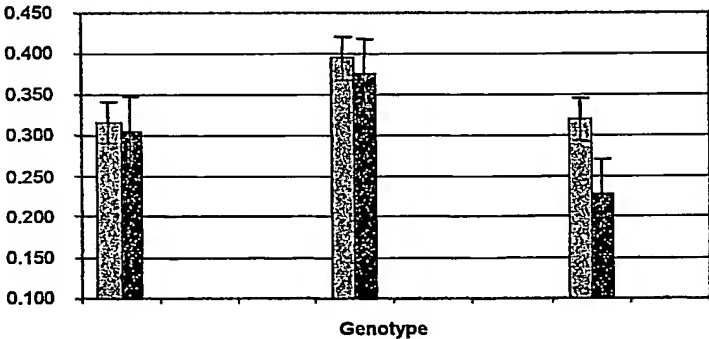


Figure 6c: Stomatal conductance
(mol H₂O m⁻²s⁻¹)



T2(+ER)
Ld_ER

Ld -er1

Ld_ER
(line 3177)

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Figure 7a: Transpiration efficiency ($\text{mmol C mol H}_2\text{O}^{-1}$) at 350 and 500ppm CO_2

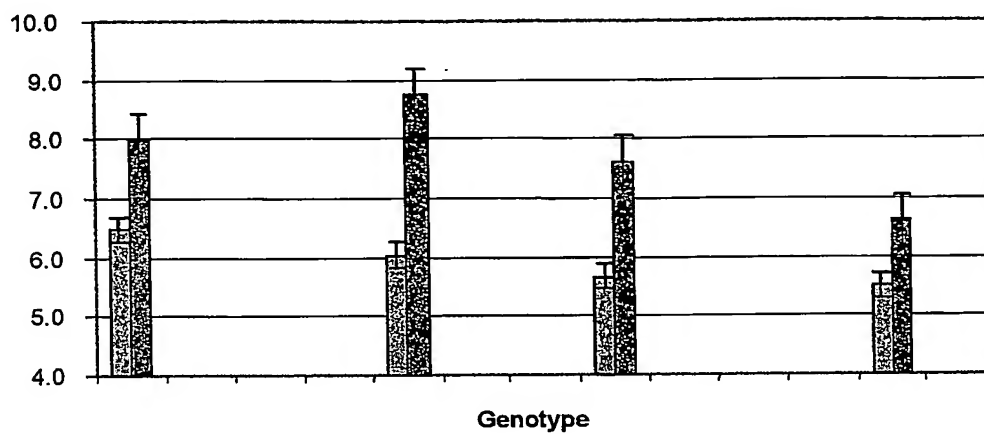


Figure 7b: CO_2 assimilation rate ($\mu\text{mol C m}^{-2}\text{s}^{-1}$)

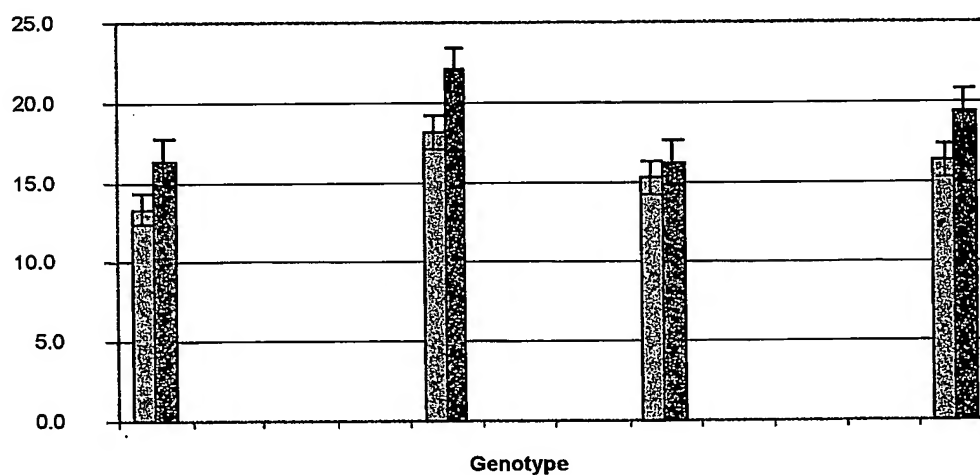
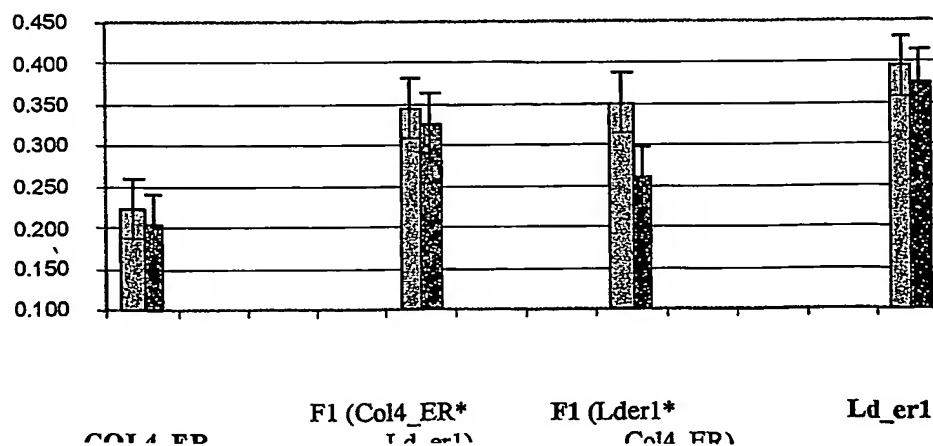


Figure 7c: Stomatal conductance ($\text{mol H}_2\text{O m}^{-2}\text{s}^{-1}$)



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Figure 8a

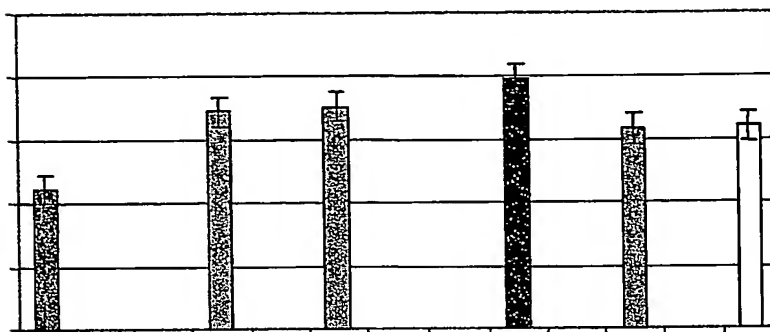
Stomatal conductance ($\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$)

Figure 8b

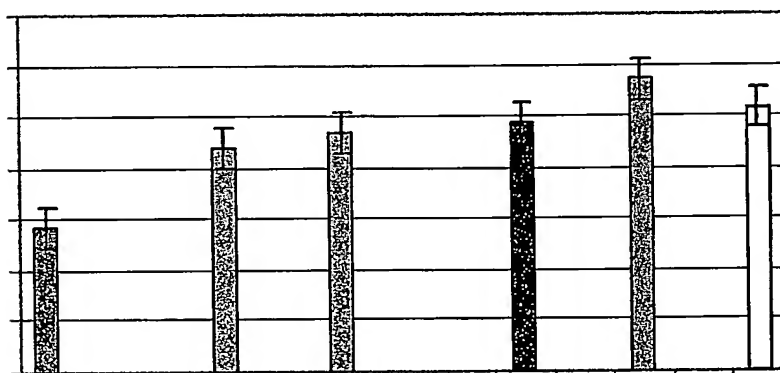
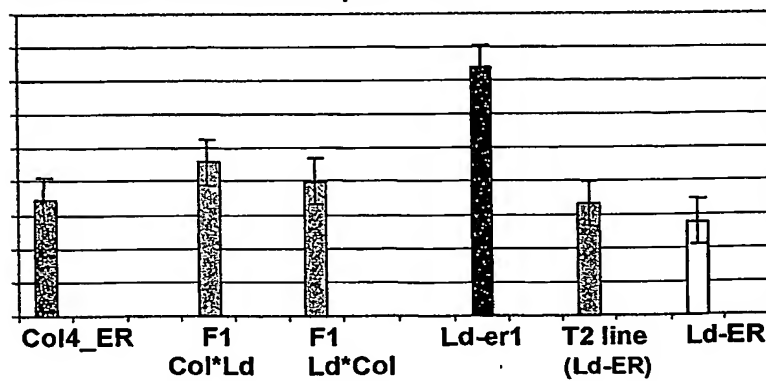
Epidermal cell area (μm^2)

Figure 8c

Number of stomata per mm^2 

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Figure 9a

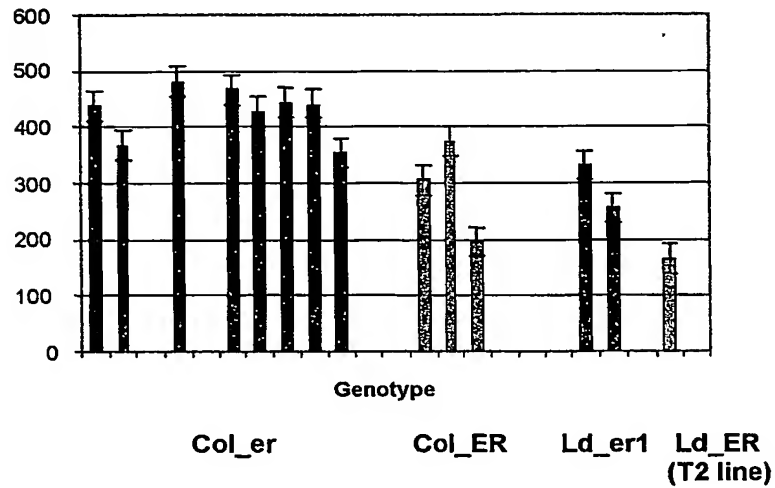
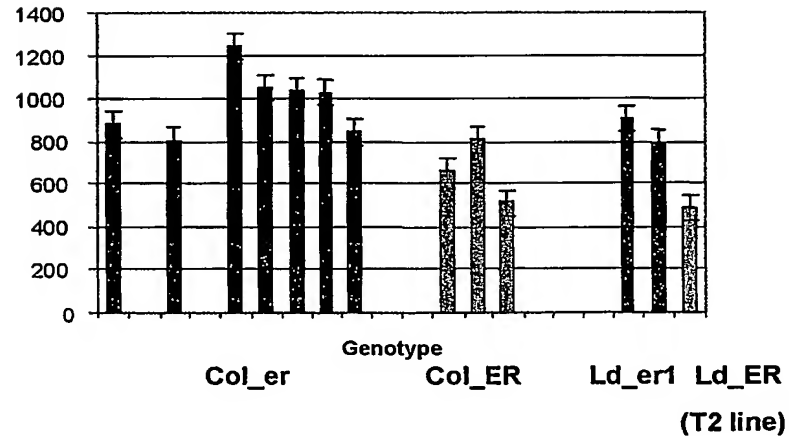
Number of stomata per mm²

Figure 9b

Epidermal cell area (μm²)

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Carbon isotope composition (per mil) for a range of er mutants
and ER ecotypes or transgenic lines

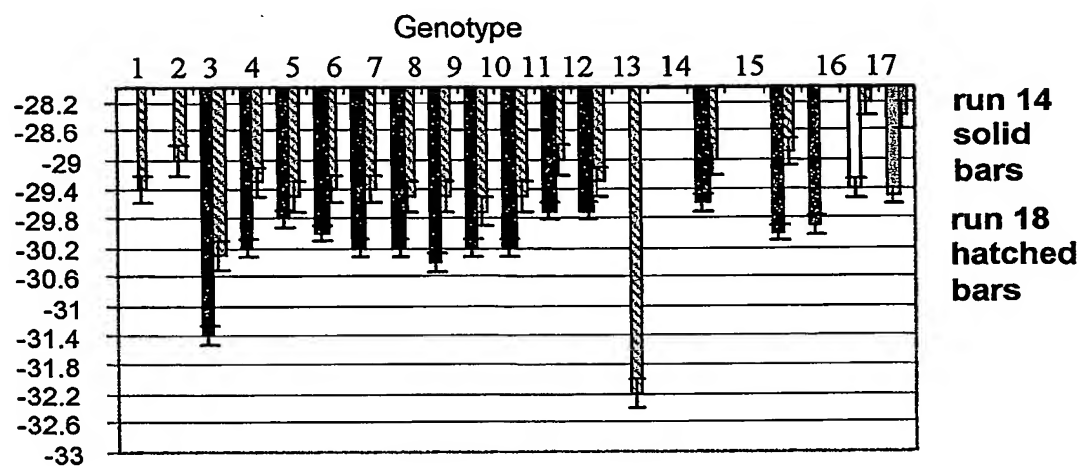


Figure 10

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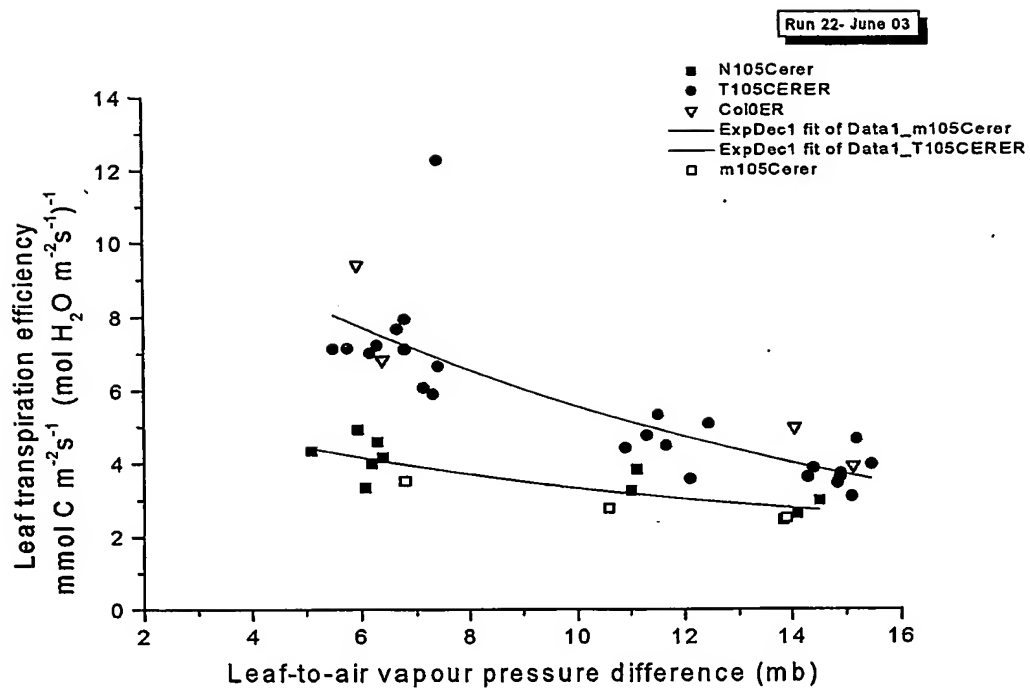


Figure 11

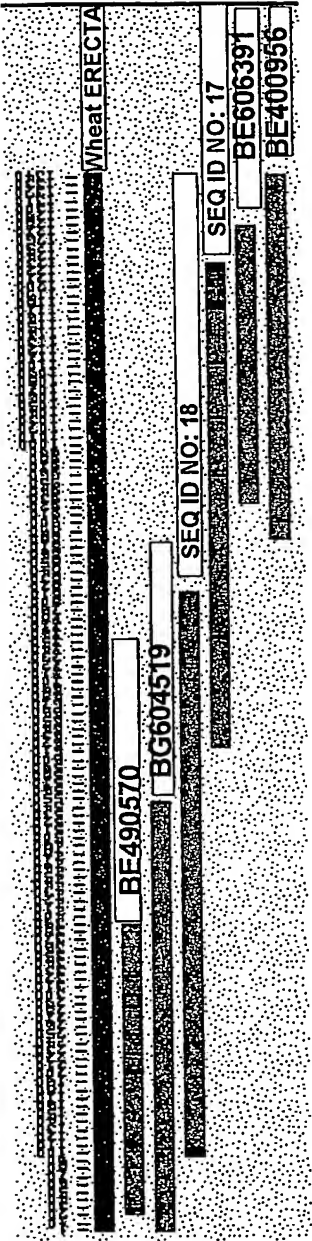
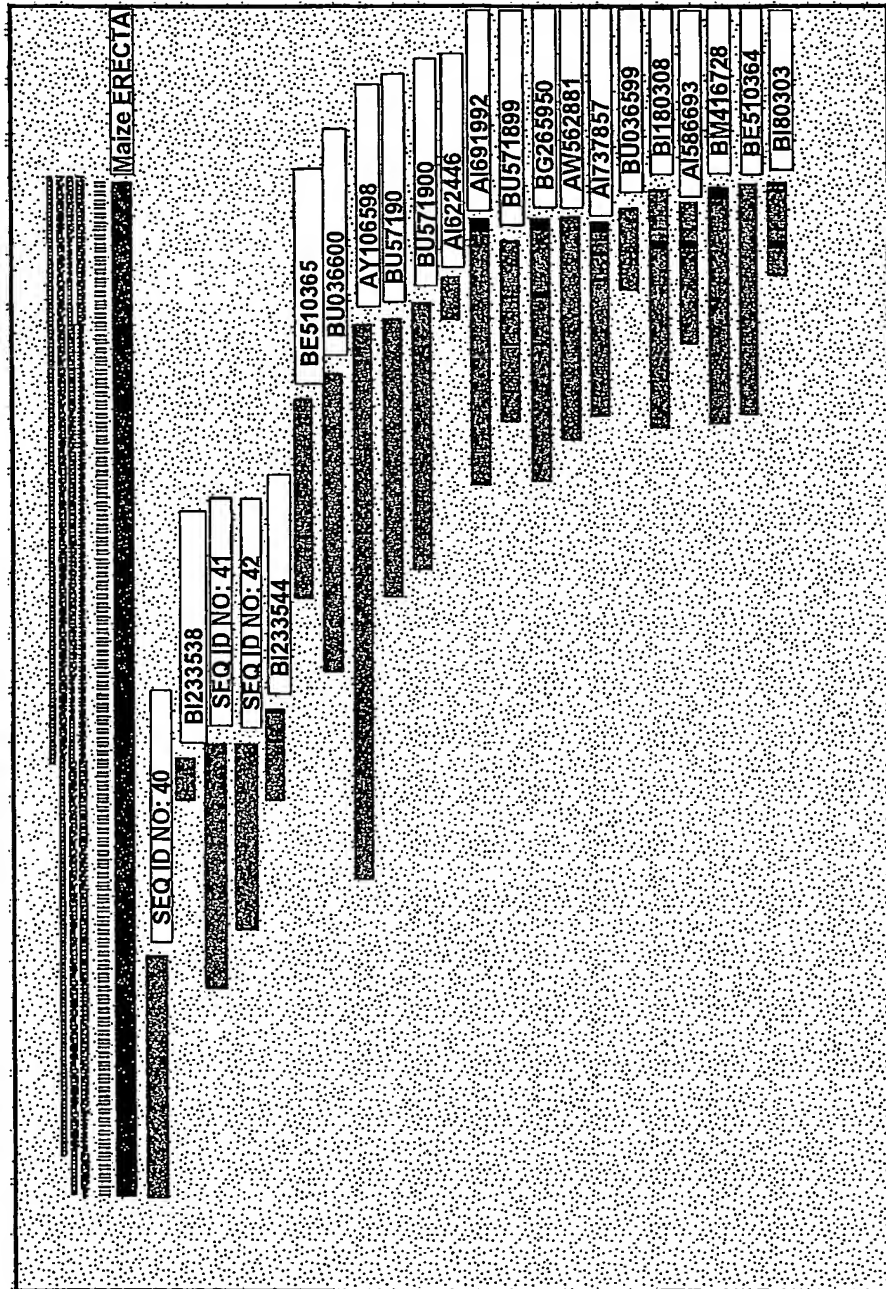


Figure 12



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Figure 13

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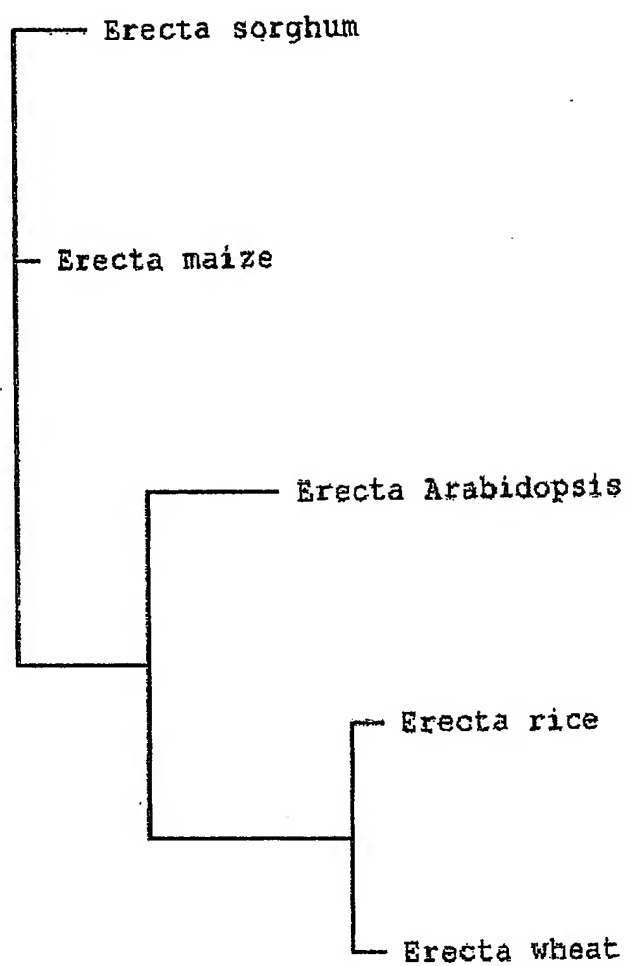


FIGURE 14

SEQUENCE LISTING

<110> The Australian National University

5 <120> METHOD OF PRODUCING PLANTS HAVING ENHANCED TRANSPIRATION EFFICIENCY AND
PLANTS PRODUCED THEREFROM

<130> 94948/MRO

10 <150> AU PS3339
<151> 2002-07-02

<160> 45

15 <170> PatentIn version 3.1

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20 <213> Arabidopsis thaliana ERECTA allele

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cttgagctta gtagctactg tgacttcaga ggaggagca acgttgctgg agattaagaa 180

gtcattcaaa gatgtgaaca atgttcttta tgactggaca acttcacctt cttcggatta 240

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gctaagtagc atcaagcaca gaaatcttgt gagcctacaa gcttattccc tctctcactt 2220

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Thr Ser Pro Ser Ser Asp Tyr Cys Val Trp Arg Gly Val Ser Cys Glu
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Asn Val Thr Phe Asn Val Val Ala Leu Asn Leu Ser Asp Leu Asn Leu
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Asp Gly Glu Ile Ser Pro Ala Ile Gly Asp Leu Lys Ser Leu Leu Ser
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Ile Asp Leu Arg Gly Asn Arg Leu Ser Gly Gln Ile Pro Asp Glu Ile
100 105 110

Gly Asp Cys Ser Ser Leu Gln Asn Leu Asp Leu Ser Phe Asn Glu Leu
115 120 125

Ser Gly Asp Ile Pro Phe Ser Ile Ser Lys Leu Lys Gln Leu Glu Gln
130 135 140

Leu Ile Leu Lys Asn Asn Gln Leu Ile Gly Pro Ile Pro Ser Thr Leu
145 150 155 160

5 Ser Gln Ile Pro Asn Leu Lys Ile Leu Asp Leu Ala Gln Asn Lys Leu
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10 Ser Gly Glu Ile Pro Arg Leu Ile Tyr Trp Asn Glu Val Leu Gln Tyr
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15 Leu Gly Leu Arg Gly Asn Asn Leu Val Gly Asn Ile Ser Pro Asp Leu
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20 Cys Gln Leu Thr Gly Leu Trp Tyr Phe Asp Val Arg Asn Asn Ser Leu
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Thr Gly Ser Ile Pro Glu Thr Ile Gly Asn Cys Thr Ala Phe Gln Val
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25 Leu Asp Leu Ser Tyr Asn Gln Leu Thr Gly Glu Ile Pro Phe Asp Ile
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30 Gly Phe Leu Gln Val Ala Thr Leu Ser Leu Gln Gly Asn Gln Leu Ser
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35 Gly Lys Ile Pro Ser Val Ile Gly Leu Met Gln Ala Leu Ala Val Leu
275 280 285

40 Asp Leu Ser Gly Asn Leu Leu Ser Gly Ser Ile Pro Pro Ile Leu Gly
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Asn Leu Thr Phe Thr Glu Lys Leu Tyr Leu His Ser Asn Lys Leu Thr
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Gly Ser Ile Pro Pro Glu Leu Gly Asn Met Ser Lys Leu His Tyr Leu
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10 Lys Leu Thr Asp Leu Phe Asp Leu Asn Val Ala Asn Asn Asp Leu Glu
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Gly Pro Ile Pro Asp His Leu Ser Ser Cys Thr Asn Leu Asn Ser Leu
15 370 375 380

Asn Val His Gly Asn Lys Phe Ser Gly Thr Ile Pro Arg Ala Phe Gln
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Lys Leu Glu Ser Met Thr Tyr Leu Asn Leu Ser Ser Asn Asn Ile Lys
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Gly Pro Ile Pro Val Glu Leu Ser Arg Ile Gly Asn Leu Asp Thr Leu
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30 Asp Leu Ser Asn Asn Lys Ile Asn Gly Ile Ile Pro Ser Ser Leu Gly
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Asp Leu Glu His Leu Leu Lys Met Asn Leu Ser Arg Asn His Ile Thr
35 450 455 460

Gly Val Val Pro Gly Asp Phe Gly Asn Leu Arg Ser Ile Met Glu Ile
465 470 475 480

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485 490 495

Gln Leu Gln Asn Ile Ile Leu Leu Arg Leu Glu Asn Asn Asn Leu Thr
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Gly Asn Val Gly Ser Leu Ala Asn Cys Leu Ser Leu Thr Val Leu Asn
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Val Ser His Asn Asn Leu Val Gly Asp Ile Pro Lys Asn Asn Asn Phe
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Ser Arg Phe Ser Pro Asp Ser Phe Ile Gly Asn Pro Gly Leu Cys Gly
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Ser Trp Leu Asn Ser Pro Cys His Asp Ser Arg Arg Thr Val Arg Val
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Ser Ile Ser Arg Ala Ala Ile Leu Gly Ile Ala Ile Gly Gly Leu Val
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Ile Leu Leu Met Val Leu Ile Ala Ala Cys Arg Pro His Asn Pro Pro
595 600 605

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Pro Phe Leu Asp Gly Ser Leu Asp Lys Pro Val Thr Tyr Ser Thr Pro
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Lys Leu Val Ile Leu His Met Asn Met Ala Leu His Val Tyr Glu Asp
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Ile Met Arg Met Thr Glu Asn Leu Ser Glu Lys Tyr Ile Ile Gly His
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Gly Ala Ser Ser Thr Val Tyr Lys Cys Val Leu Lys Asn Cys Lys Pro

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5 Val Ala Ile Lys Arg Leu Tyr Ser His Asn Pro Gln Ser Met Lys Gln
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10 Phe Glu Thr Glu Leu Glu Met Leu Ser Ser Ile Lys His Arg Asn Leu
690 695 700

15 Val Ser Leu Gln Ala Tyr Ser Leu Ser His Leu Gly Ser Leu Leu Phe
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20 Tyr Asp Tyr Leu Glu Asn Gly Ser Leu Trp Asp Leu Leu His Gly Pro
725 730 735

Thr Lys Lys Lys Thr Leu Asp Trp Asp Thr Arg Leu Lys Ile Ala Tyr
740 745 750

25 Gly Ala Ala Gln Gly Leu Ala Tyr Leu His His Asp Cys Ser Pro Arg
755 760 765

30 Ile Ile His Arg Asp Val Lys Ser Ser Asn Ile Leu Leu Asp Lys Asp
770 775 780

Leu Glu Ala Arg Leu Thr Asp Phe Gly Ile Ala Lys Ser Leu Cys Val
785 790 795 800

35 Ser Lys Ser His Thr Ser Thr Tyr Val Met Gly Thr Ile Gly Tyr Ile
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40 Asp Pro Glu Tyr Ala Arg Thr Ser Arg Leu Thr Glu Lys Ser Asp Val
820 825 830

Tyr Ser Tyr Gly Ile Val Leu Leu Glu Leu Leu Thr Arg Arg Lys Ala
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5 Val Asp Asp Glu Ser Asn Leu His His Leu Ile Met Ser Lys Thr Gly
 850 855 860

Asn Asn Glu Val Met Glu Met Ala Asp Pro Asp Ile Thr Ser Thr Cys
 10 865 870 875 880

Lys Asp Leu Gly Val Val Lys Lys Val Phe Gln Leu Ala Leu Leu Cys
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15 Thr Lys Arg Gln Pro Asn Asp Arg Pro Thr Met His Gln Val Thr Arg
 900 905 910

20 Val Leu Gly Ser Phe Met Leu Ser Glu Gln Pro Pro Ala Ala Thr Asp
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25 Thr Ser Ala Thr Leu Ala Gly Ser Cys Tyr Val Asp Glu Tyr Ala Asn
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Leu Lys Thr Pro His Ser Val Asn Cys Ser Ser Met Ser Ala Ser Asp
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Ala Ala Asn Ala Leu Val Asp Trp Asp Gly Gly Ala Asp His Cys Ala
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Trp Arg Gly Val Thr Cys Asp Asn Ala Ser Phe Ala Val Leu Ala Leu
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Asn Leu Ser Asn Leu Asn Leu Gly Gly Glu Ile Ser Pro Ala Ile Gly
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5 Glu Leu Lys Asn Leu Gln Phe Val Asp Leu Lys Gly Asn Lys Leu Thr
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10 Gly Gln Ile Pro Asp Glu Ile Gly Asp Cys Ile Ser Leu Lys Tyr Leu
 115 120 125

15 Asp Leu Ser Gly Asn Leu Leu Tyr Gly Asp Ile Pro Phe Ser Ile Ser
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20 Lys Leu Lys Gln Leu Glu Glu Leu Ile Leu Lys Asn Asn Gln Leu Thr
 145 150 155 160

25 Gly Pro Ile Pro Ser Thr Leu Ser Gln Ile Pro Asn Leu Lys Thr Leu
 165 170 175

30 Asp Leu Ala Gln Asn Gln Leu Thr Gly Asp Ile Pro Arg Leu Ile Tyr
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35 Trp Asn Glu Val Leu Gln Tyr Leu Gly Leu Arg Gly Asn Ser Leu Thr
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40 Gly Thr Leu Ser Pro Asp Met Cys Gln Leu Thr Gly Leu Trp Tyr Phe
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45 Asp Val Arg Gly Asn Asn Leu Thr Gly Thr Ile Pro Glu Ser Ile Gly
 225 230 235 240

50 Asn Cys Thr Ser Phe Glu Ile Leu Asp Ile Ser Tyr Asn Gln Ile Ser
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55 Gly Glu Ile Pro Tyr Asn Ile Gly Phe Leu Gln Val Ala Thr Leu Ser

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40 Leu Ser Ser Asn Asn Phe Lys Gly Asn Ile Pro Ser Glu Leu Gly His
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Ile Ile Asn Leu Asp Thr Leu Asp Leu Ser Tyr Asn Glu Phe Ser Gly
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5 Pro Val Pro Ala Thr Ile Gly Asp Leu Glu His Leu Leu Glu Leu Asn
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Leu Ser Lys Asn His Leu Asp Gly Pro Val Pro Ala Glu Phe Gly Asn
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Leu Arg Ser Val Gln Val Ile Asp Met Ser Asn Asn Asn Leu Ser Gly
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15 Ser Leu Pro Glu Glu Leu Gly Gln Leu Gln Asn Leu Asp Ser Leu Ile
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20 Leu Asn Asn Asn Asn Leu Val Gly Glu Ile Pro Ala Gln Leu Ala Asn
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25 Cys Phe Ser Leu Asn Asn Leu Ala Phe Gln Glu Phe Val Ile Gln Gln
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Phe Ile Trp Thr Cys Pro Asp Gly Lys Glu Leu Leu Glu Ile Pro Asn
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Gly Lys His Leu Leu Ile Ser Asp Cys Asn Gln Tyr Ile Asn His Lys
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35 Cys Ser Phe Leu Gly Asn Pro Leu Leu His Val Tyr Cys Gln Asp Ser
580 585 590

40 Ser Cys Gly His Ser His Gly Gln Arg Val Asn Ile Ser Lys Thr Ala
595 600 605

Ile Ala Cys Ile Ile Leu Gly Phe Ile Ile Leu Leu Cys Val Leu Leu
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Leu Ala Ile Tyr Lys Thr Asn Gln Pro Gln Pro Leu Val Lys Gly Ser
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10 Asp Lys Pro Val Gln Gly Pro Pro Lys Leu Val Val Leu Gln Met Asp
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15 Met Ala Ile His Thr Tyr Glu Asp Ile Met Arg Leu Thr Glu Asn Leu
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Ser Glu Lys Tyr Ile Ile Gly Tyr Gly Ala Ser Ser Thr Val Tyr Lys
675 680 685

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Cys Glu Leu Lys Ser Gly Lys Ala Ile Ala Val Lys Arg Leu Tyr Ser
690 695 700

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Gln Tyr Asn His Ser Leu Arg Glu Phe Glu Thr Glu Leu Glu Thr Ile
705 710 715 720

30 Gly Ser Ile Arg His Arg Asn Leu Val Ser Leu His Gly Phe Ser Leu
725 730 735

Ser Pro His Gly Asn Leu Leu Phe Tyr Asp Tyr Met Glu Asn Gly Ser
35 740 745 750

Leu Trp Asp Leu Leu His Gly Pro Ser Lys Lys Val Lys Leu Asn Trp
755 760 765

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Asp Thr Arg Leu Arg Ile Ala Val Gly Ala Ala Gln Gly Leu Ala Tyr
770 775 780

Leu His His Asp Cys Asn Pro Arg Ile Ile His Arg Asp Val Lys Ser
785 790 795 800

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Ser Asn Ile Leu Leu Asp Glu Asn Phe Glu Ala His Leu Ser Asp Phe
805 810 815

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Gly Ile Ala Lys Cys Val Pro Ser Ala Lys Ser His Ala Ser Thr Tyr
820 825 830

15 Val Leu Gly Thr Ile Gly Tyr Ile Asp Pro Glu Tyr Ala Arg Thr Ser
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Arg Leu Asn Glu Lys Ser Asp Val Tyr Ser Phe Gly Ile Val Leu Leu
20 850 855 860

Glu Leu Leu Thr Gly Lys Lys Ala Val Asp Asn Glu Ser Asn Leu His
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Gln Leu Ile Leu Ser Lys Ala Asp Asp Asn Thr Val Met Glu Ala Val
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Asp Ser Glu Val Ser Val Thr Cys Thr Asp Met Gly Leu Val Arg Lys
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35 Ala Phe Gln Leu Ala Leu Leu Cys Thr Lys Arg His Pro Ser Asp Arg
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Pro Thr Met His Glu Val Ala Arg Val Leu Leu Ser Leu Leu Pro Ala
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Ser Ala Met Thr Thr Pro Lys Thr Val Asp Tyr Ser Arg Leu Leu Ala

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Cys Ser Trp Arg Gly Val Leu Cys Asp Asn Val Thr Phe Ala Val Ala
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Ala Leu Asn Leu Ser Gly Leu Asn Leu Glu Gly Glu Ile Ser Pro Ala
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15 Val Gly Ser Leu Lys Ser Leu Val Ser Ile Asp Leu Lys Ser Asn Gly
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Leu Ser Gly Gln Ile Pro Asp Glu Ile Gly Asp Cys Ser Ser Leu Arg
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Thr Leu Asp Phe Ser Phe Asn Asn Leu Asp Gly Asp Ile Pro Phe Ser
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25

Ile Ser Lys Leu Lys His Leu Glu Asn Leu Ile Leu Lys Asn Asn Gln
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Leu Ile Gly Ala Ile Pro Ser Thr Leu Ser Gln Leu Pro Asn Leu Lys
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35 Ile Leu Asp Leu Ala Gln Asn Lys Leu Thr Gly Glu Ile Pro Arg Leu
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Ile Tyr Trp Asn Glu Val Leu Gln Tyr Leu Asp Val Lys Asn Asn Ser
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Leu Thr Gly Val Ile Pro Asp Thr Ile Gly Asn Cys Thr Ser Phe Gln

22

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Ile Gly Phe Leu Gln Val Ala Thr Leu Ser Leu Gln Gly Asn Lys Phe
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Thr Gly Pro Ile Pro Ser Val Ile Gly Leu Met Gln Ala Leu Ala Val
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Leu Asp Leu Ser Tyr Asn Gln Leu Ser Gly Pro Ile Pro Ser Ile Leu
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20 Gly Asn Leu Thr Tyr Thr Glu Lys Leu Tyr Ile Gln Gly Asn Lys Leu
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Thr Gly Ser Ile Pro Pro Glu Leu Gly Asn Met Ser Thr Leu His Tyr
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Leu Glu Leu Asn Asp Asn Gln Leu Thr Gly Ser Ile Pro Pro Glu Leu
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Gly Arg Leu Thr Gly Leu Phe Asp Leu Asn Leu Ala Asn Asn His Leu
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40 Phe Asn Ala Tyr Gly Asn Lys Leu Asn Gly Thr Ile Pro Arg Ser Leu
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Arg Lys Leu Glu Ser Met Thr Tyr Leu Asn Leu Ser Ser Asn Phe Ile
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5 Ser Gly Ser Ile Pro Ile Glu Leu Ser Arg Ile Asn Asn Leu Asp Thr
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Ser Phe Leu Gly Asn Pro Gly Leu Cys Gly Tyr Trp Leu Gly Ser Ser
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10 Met Asn Met Ala Leu His Val Tyr Asp Asp Ile Met Arg Met Thr Glu
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Asn Leu Ser Glu Lys Tyr Ile Ile Gly Tyr Gly Ala Ser Ser Thr Val
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Tyr Lys Cys Val Leu Lys Asn Cys Lys Pro Val Ala Ile Lys Lys Leu
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Tyr Ala His Tyr Pro Gln Ser Leu Lys Glu Phe Glu Thr Glu Leu Glu
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Thr Val Gly Ser Ile Lys His Arg Asn Leu Val Ser Leu Gln Gly Tyr
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30 Ser Leu Ser Pro Val Gly Asn Leu Leu Phe Tyr Asp Tyr Met Glu Cys
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Gly Ser Leu Trp Asp Val Leu His Glu Gly Ser Ser Lys Lys Lys Lys
35 675 680 685

Leu Asp Trp Glu Thr Arg Leu Arg Ile Ala Leu Gly Ala Ala Gln Gly
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Leu Ala Tyr Leu His His Asp Cys Ser Pro Arg Ile Ile His Arg Asp
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Val Lys Ser Lys Asn Ile Leu Leu Asp Lys Asp Tyr Glu Ala His Leu
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Thr Asp Phe Gly Ile Ala Lys Ser Leu Cys Val Ser Lys Thr His Thr
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Ser Thr Tyr Val Met Gly Thr Ile Gly Tyr Ile Asp Pro Glu Tyr Ala
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15 Arg Thr Ser Arg Leu Asn Glu Lys Ser Asp Val Tyr Arg Leu Trp His
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Cys Ser Ala Gly Ala Ala Asp Trp Gln Glu Ala Ser Gly Gln Arg Ile
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Leu Ser Lys Thr Ala Ser Asn Glu Val Met Asp Thr Val Asp Pro Asp
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Ile Gly Asp Thr Cys Lys Asp Leu Gly Glu Val Lys Lys Leu Phe Gln
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Leu Ala Leu Leu Cys Thr Lys Arg Gln Pro Ser Asp Arg Pro Thr Met
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35 His Glu Val Val Arg Val Leu Asp Cys Leu Val Asn Pro Asp Pro Pro
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Pro Lys Pro Ser Ala His Gln Leu Pro Gln Pro Ser Pro Ala Val Pro
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Ser Tyr Ile Asn Glu Tyr Val Ser Leu Arg Gly Thr Gly Ala Leu Ser

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40 Cys Asp Asn Val Ser Leu Asn Val Val Ser Leu Asn Leu Ser Asn Leu
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5 Gln Ser Ile Asp Leu Gln Gly Asn Lys Leu Gly Gly Gln Ile Pro Asp
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Glu Ile Gly Asn Cys Val Ser Leu Ala Tyr Val Asp Phe Ser Thr Asn
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Leu Leu Phe Gly Asp Ile Pro Phe Ser Ile Ser Lys Leu Lys Gln Leu
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Glu Phe Leu Asn Leu Lys Asn Asn Gln Leu Thr Gly Pro Ile Pro Ala
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Thr Leu Thr Gln Ile Pro Asn Leu Lys Thr Leu Asp Leu Ala Arg Asn
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25 Gln Leu Thr Gly Glu Ile Pro Arg Leu Leu Tyr Trp Asn Glu Val Leu
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Gln Tyr Leu Gly Leu Arg Gly Asn Met Leu Thr Gly Thr Leu Ser Pro
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Asp Met Cys Gln Leu Thr Gly Leu Trp Tyr Phe Asp Val Arg Gly Asn
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Asn Leu Thr Gly Thr Ile Pro Glu Ser Ile Gly Asn Cys Thr Ser Phe
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Glu Ile Leu Asp Val Ser Tyr Asn Gln Ile Thr Gly Val Ile Pro Tyr
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Cys Ser Trp Arg Gly Val Leu Cys Asp Asn Val Thr Phe Ala Val Ala
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Ala Leu Asn Leu Ser Gly Leu Asn Leu Glu Gly Glu Ile Ser Pro Ala
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Val Gly Ser Leu Lys Ser Leu Val Ser Ile Asp Leu Lys Ser Asn Gly
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Leu Ser Gly Gln Ile Pro Asp Glu Ile Gly Asp Cys Ser Ser Leu Arg
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Thr Leu Asp Phe Ser Phe Asn Asn Leu Asp Gly Asp Ile Pro Phe Ser
115 120 125
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Ile Ser Lys Leu Lys His Leu Glu Asn Leu Ile Leu Lys Asn Asn Gln
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Leu Ile Gly Ala Ile Pro Ser Thr Leu Ser Gln Leu Pro Asn Leu Lys
145 150 155 160
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Ile Leu Asp Leu Ala Gln Asn Lys Leu Thr Gly Glu Ile Pro Arg Leu
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Ile Tyr Trp Asn Glu Val Leu Gln Tyr Leu Asp Val Lys Asn Asn Ser
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Leu Thr Gly Val Ile Pro Asp Thr Ile Gly Asn Cys Thr Ser Phe Gln

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Arg Lys Leu Glu Ser Met Thr Tyr Leu Asn Leu Ser Ser Asn Phe Ile
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Ile Asp Leu Ser Tyr Asn His Leu Gly Gly Leu Ile Pro Gln Glu Leu
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25 Glu Met Leu Gln Asn Leu Met Leu Leu Asn Val Ser Tyr Asn Asn Leu
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Ala Gly Val Val Pro Ala Asp Asn Asn Phe Thr Arg Phe Ser Pro Asp
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Ser Phe Leu Gly Asn Pro Gly Leu Cys Gly Tyr Trp Leu Gly Ser Ser
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Cys Arg Ser Thr Gly His His Glu Lys Pro Pro Ile Ser Lys Ala Ala
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Ile Ile Gly Val Ala Val Gly Gly Leu Val Ile Leu Leu Met Ile Leu
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Asn Ile Gly Phe Leu Gln Val Ala Thr Leu Ser Leu Gln Gly Asn Lys
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15 Leu Gly Asn Leu Ser Phe Thr Gly Lys Leu Tyr Leu His Gly Asn Lys
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20 Leu Thr Gly Gln Ile Pro Pro Glu Leu Gly Asn Met Ser Arg Leu Ser
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Tyr Leu Gln Leu Asn Asp Asn Glu Leu Val Gly Lys Ile Pro Pro Glu
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Thr Leu Asp Leu Ser Gly Asn Asn Phe Ser Gly Ser Ile Pro Leu Thr
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Leu Gly Asp Leu Glu His Leu Leu Ile Leu Asn Leu Ser Arg Asn His
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Leu Asn Gly Thr Leu Pro Ala Glu Phe Gly Asn Leu Arg Ser Ile Gln
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Lys Asn Phe Thr Arg Phe Ser Pro Ala Ser Phe Phe Gly Asn Pro Phe
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Ser Gln Val Phe Thr Arg Val Ala Val Ile Cys Met Val Leu Gly Phe
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Ile Thr Leu Ile Cys Met Ile Phe Ile Ala Val Tyr Lys Ser Lys Gln

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40 Gly Ala Ala Gln Gly Leu Ala Tyr Leu His His Asp Cys Thr Pro Arg
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Ile Ile His Arg Asp Ile Lys Ser Ser Asn Ile Leu Leu Asp Gly Asn
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5 Phe Glu Ala Arg Leu Ser Asp Phe Gly Ile Ala Lys Ser Ile Pro Ala
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Thr Lys Thr Tyr Ala Ser Thr Tyr Val Leu Gly Thr Ile Gly Tyr Ile
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Tyr Ser Phe Gly Ile Val Leu Leu Glu Leu Leu Thr Gly Lys Lys Ala
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Val Leu Leu Ser Leu Val Pro Ser Pro Pro Pro Lys Lys Leu Pro Ser
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5 Asp Trp Asp Asp Val His Asn Ser Asp Leu Cys Ser Trp Arg Gly Val
50 55 60

Phe Cys Asp Asn Val Ser Tyr Ser Val Val Ser Leu Asn Leu Ser Ser
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Leu Asn Leu Gly Gly Glu Ile Ser Pro Ala Ile Gly Asp Leu Arg Asn
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Leu Gln Ser Ile Asp Leu Gln Gly Asn Lys Leu Ala Gly Gln Ile Pro
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20 Asp Glu Ile Gly Asn Cys Ala Ser Leu Val Tyr Leu Asp Leu Ser Glu
115 120 125

25 Asn Leu Leu Tyr Gly Asp Ile Pro Phe Ser Ile Ser Lys Leu Lys Gln
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Leu Glu Thr Leu Asn Leu Lys Asn Asn Gln Leu Thr Gly Pro Val Pro
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Ala Thr Leu Thr Gln Ile Pro Asn Leu Lys Arg Leu Asp Leu Ala Gly
165 170 175
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Asn His Leu Thr Gly Glu Ile Ser Arg Leu Leu Tyr Trp Asn Glu Val
180 185 190

40 Leu Gln Tyr Leu Gly Leu Arg Gly Asn Met Leu Thr Gly Thr Leu Ser
195 200 205

Ser Asp Met Cys Gln Leu Thr Gly Leu Trp Tyr Phe Asp Val Arg Gly
210 215 220

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10 Phe Gln Ile Leu Asp Ile Ser Tyr Asn Gln Ile Thr Gly Glu Ile Pro
245 250 255

15 Tyr Asn Ile Gly Phe Leu Gln Val Ala Thr Leu Ser Leu Gln Gly Asn
260 265 270

20 Arg Leu Thr Gly Arg Ile Pro Glu Val Ile Gly Leu Met Gln Ala Leu
275 280 285

Ala Val Leu Asp Leu Ser Asp Asn Glu Leu Val Gly Pro Ile Pro Pro
290 295 300

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Ile Leu Gly Asn Leu Ser Phe Thr Gly Lys Leu Tyr Leu His Gly Asn
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30 Met Leu Thr Gly Pro Ile Pro Ser Glu Leu Gly Asn Met Ser Arg Leu
325 330 335

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340 345 350

Glu Leu Gly Lys Leu Glu Gln Leu Phe Glu Leu Asn Leu Ala Asn Asn
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Arg Leu Val Gly Pro Ile Pro Ser Asn Ile Ser Ser Cys Ala Ala Leu
370 375 380

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385 390 395 400
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Ala Phe Arg Asn Leu Gly Ser Leu Thr Tyr Leu Asn Leu Ser Ser Asn
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Asn Phe Lys Gly Lys Ile Pro Val Glu Leu Gly His Ile Ile Asn Leu
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Asp Lys Leu Asp Leu Ser Gly Asn Asn Phe Ser Gly Ser Ile Pro Leu
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Thr Leu Gly Asp Leu Glu His Leu Leu Ile Leu Asn Leu Ser Arg Asn
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His Leu Ser Gly Gln Leu Pro Ala Glu Phe Gly Asn Leu Arg Ser Ile
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Gln Met Ile Asp Val Ser Phe Asn Leu Leu Ser Gly Val Ile Pro Thr
485 490 495

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Lys Leu His Gly Lys Ile Pro Asp Gln Leu Thr Asn Cys Phe Thr Leu
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Val Asn Leu Asn Val Ser Phe Asn Asn Leu Ser Gly Ile Val Pro Pro
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Met Lys Asn Phe Ser Arg Phe Ala Pro Ala Ser Phe Val Gly Asn Pro

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Ser Arg Val Phe Ser Arg Gly Ala Leu Ile Cys Ile Val Leu Gly Val			
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Ile Thr Leu Leu Cys Met Ile Phe Leu Ala Val Tyr Lys Ser Met Gln			
	595	600	605
15	Gln Lys Lys Ile Leu Gln Gly Ser Ser Lys Gln Ala Glu Gly Leu Thr		
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20	Lys Leu Val Ile Leu His Met Asp Met Ala Ile His Thr Phe Asp Asp		
	625	630	635
	Ile Met Arg Val Thr Glu Asn Leu Asn Glu Lys Phe Ile Ile Gly Tyr		
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Gly Ala Ser Ser Thr Val Tyr Lys Cys Ala Leu Lys Ser Ser Arg Pro			
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Ile Ala Ile Lys Arg Leu Tyr Asn Gln Tyr Pro His Asn Leu Arg Glu			
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35	Phe Glu Thr Glu Leu Glu Thr Ile Gly Ser Ile Arg His Arg Asn Ile		
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40	Val Ser Leu His Gly Tyr Ala Leu Ser Pro Thr Gly Asn Leu Leu Phe		
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			720

41

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725 730 735

5 Leu Lys Lys Val Lys Leu Asp Trp Glu Thr Arg Leu Lys Ile Ala Val
740 745 750

Gly Ala Ala Gln Gly Leu Ala Tyr Leu His His Asp Cys Thr Pro Arg
10 755 760 765

Ile Ile His Arg Asp Ile Lys Ser Ser Asn Ile Leu Leu Asp Glu Asn
15 770 775 780

Phe Glu Ala His Leu Ser Asp Phe Gly Ile Ala Lys Ser Ile Pro Ala
785 790 795 800

20 Ser Lys Thr His Ala Ser Thr Tyr Val Leu Gly Thr Ile Gly Tyr Ile
805 810 815

25 Asp Pro Glu Tyr Ala Arg Thr Ser Arg Ile Asn Glu Lys Ser Asp Ile
820 825 830

Tyr Ser Phe Gly Ile Val Leu Leu Glu Leu Leu Thr Gly Lys Lys Ala
30 835 840 845

Val Asp Asn Glu Ala Asn Leu His Gln Leu Ile Leu Ser Lys Ala Asp
35 850 855 860

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40 Met Asp Leu Gly His Ile Arg Lys Thr Phe Gln Leu Ala Leu Leu Cys
885 890 895

Thr Lys Arg Asn Pro Leu Glu Arg Pro Thr Met Leu Glu Val Ser Arg
 900 905 910

5

Val Leu Leu Ser Leu Val Pro Ser Leu Gln Val Ala Lys Lys Leu Pro
 915 920 925

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Ser Leu Asp His Ser Thr Lys Lys Leu Gln Gln Glu Asn Glu Val Arg
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20 cctgctacta ttggtgatct tgagcatctt cttcaactaa atttgagcaa aaaccatctt 240

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25 ttgattctta acaacaatat tttggctgga gagatccctg ctgagttggc taactgcttc 420

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25 gctactattg gtgatcttga gcatcttctt caactaaatt tgagcaaaaa ccatcttagc 180

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25 cggtgcggca caagggtgg catatcttca ccatgactgc aaccctcgga tagtcacag 360

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30 cggcatcggc aaatgcgtcc cagctgccc aacccacgcg tccacctatg tgctaggaac 480

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 Lys Gly His Ile Pro Ser Glu Leu Gly His Ile Ile Asn Leu Asp Thr
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49

Gly Asp Leu Glu His Leu Leu Gln Leu Asn Leu Ser Lys Asn His Leu
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10 Ile Asp Leu Ser Asn Asn Ala Met Ser Gly Tyr Leu Pro Glu Glu Leu
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15 Gly Gln Leu Gln Asn Leu Asp Ser Leu Ile Leu Asn Asn Asn Ile Leu
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Val Gly Glu Ile Pro Ala Gln Leu Ala Asn Cys Phe Ser Leu Asn Ile
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20 Leu Asn Leu Ser His Asn Asn Phe Ser Gly His Val Pro Phe Ala Lys
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25 Asn Phe Ser Lys Phe Pro Gly Glu Ser Phe Leu Gly Asn Pro Met Leu
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30 Ser Val His Cys Lys Asp Ser Ser Cys Gly Asn Ser His Gly Ser Lys
 180 185 190

35 Val Asn Thr Arg Thr Ala Ile Ala Cys Ile Ile Ser Gly Phe Val Ile
 195 200 205

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40 Pro Pro Ile Lys Ala Ser Asp Lys Pro Gly Gln Gly Pro Pro Lys Ile
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Thr Glu Leu Glu Thr Val Gly Ser Ile Arg His Arg Asn Leu Val Ser
305 310 315 320

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325 330 335

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340 345 350

30 Lys Val Lys Leu Asp Trp Asp Thr Arg Leu Arg Ile Ala Val Gly Ala
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Ala Gln Gly Leu Ala Tyr Leu His His Asp Cys Asn Pro Arg Ile Val
35 370 375 380

His Arg Asp Val Lys Ser Ser Asn Ile Leu Leu Asp Glu His Phe Glu
385 390 395 400

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Ala His Leu Ser Asp Phe Gly Ile Ala Lys Cys Val Pro Ala Ala Lys
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Thr His Ala Ser Thr Tyr Val Leu Gly Thr Ile Gly Tyr Ile Asp Pro
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5 cgaacgtcta cgtcttcccc tacaccatgt tctgcgagat ggctcgccg aacttgagga 240

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ggctgacgta ctcgtcgacg taggccggcg gcgacggctg cggcggcagc tgggtggtgcg 360

10 cgtgcgcctt ctgctgctgc tgctgcggct gcggcgggcg ctccgggttc accaggcagt 420

caaggacgcg caccacctcg tgcctcgtcg gccgggtccga gggctgccgc ttggtgcaga 480

15 ggagcgccag ctggaacagc ttcttcacct cgcccaggtc cttgcagng tctcccacgt 540

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<212> DNA

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30 aatgtcagcc aaactactcc ctgcaatcgg cctcactcaa ggcgccctcac cgaacgtcta 180

cgtcttcccc tacaccatgt tctgcgagat ggctcgccg aacttgagga acagctcggc 240

gtccgaggtg ctggacgagt tggcgagga gaggcgccg gtgccccgca ggctgacgta 300

35 ctcgtcgacg taggccggcg gggacggctg cggcggcagc tgggtggtgcg cgtgcgcctt 360

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40 caccacctcg ggcac 436

<210> 25

<211> 509

<212> DNA

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acttgaggaa cagctcggcg tccgaggtgc tggacgagtt ggcgcaggag agggcgccgg 300

15 tgccccgcag gctgacgtac tcgtcgacgt aggccggcgg cgacggctgc ggcggcagct 360

ggtggtgcgc gtgcgccttc tgctgctgct gctgcggctg cggcggcggc tccgggttca 420

20 ccaggcagtc aaggacgcgc accacctcgt gcatcgtcgg ccggtccgag ggctgccgct 480

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<211> 318

<212> DNA

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35 accctagcta ctgagtccca tgttaatctc ctgcgctgcg tcccacaaa tgtcagtcaa 180

actactcccc ctgcaatcgg cctcactcaa ggcgcctcac cgaacgtcta cgtcttcccc 240

tacaccatgt tctgcgagat ggcctcgccg aacttgagga acagctcggc gtccgaggtg 300

40 ctggacgagt tggcgagc 318

<210> 27
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<212> DNA
<213> partial maize ERECTA

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15 <213> partial maize ERECTA

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catggagacg gtggaccccg acgtgggaga cacctgcaag gacctgggag aggtgaagaa 360

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tgctcttgcg tcacatgact ttttttacag ctaacaacac cctagctact gagtcccatg 180
30 ttaatctcct gcgctgcgtc ccacaa 206

<210> 31
35 <211> 534
<212> DNA
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tgcggtccac aaaatgtcag tcaaactact cccctgcaa tcggcctcac tcaaggcgcc 240

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20

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<211> 412

<212> DNA

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gcagctgggg gtgcgcgtgc gccttctgct gctgctgctg cggttgcggc ggcggctccg 360
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<210> 34

20 <211> 533

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20 <212> PRT

<213> maize ERECTA

<400> 45

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30 Met Ser Thr Leu His Tyr Leu Glu Leu Asn Asp Asn Gln Leu Thr Gly
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Ser Ile Pro Pro Glu Leu Gly Arg Leu Thr Gly Leu Phe Asp Leu Asn
 35 40 45

40 Leu Ala Asn Asn His Leu Glu Gly Pro Ile Pro Asp Asn Leu Ser Ser
 50 55 60

Cys Val Asn Leu Asn Ser Phe Asn Ala Tyr Gly Asn Lys Leu Asn Gly
 65 70 75 80

Thr Ile Pro Arg Ser Leu Arg Lys Leu Glu Ser Met Thr Tyr Leu Asn
85 90 95

5
Leu Ser Ser Asn Phe Ile Ser Gly Ser Ile Pro Ile Glu Leu Ser Arg
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10 Ile Asn Asn Leu Asp Thr Leu Asp Leu Ser Cys Asn Met Met Thr Gly
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Pro Ile Pro Ser Ser Ile Gly Asn Leu Glu His Leu Leu Arg Leu Asn
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Leu Ser Lys Asn Asp Leu Val Gly Phe Ile Pro Ala Glu Phe Gly Asn
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Leu Ile Pro Gln Glu Leu Gly Met Leu Gln Asn Leu Met Leu Leu Lys
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30 Leu Glu Asn Asn Asn Ile Thr Gly Asp Val Ser Ser Leu Met Asn Cys
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Phe Ser Leu Asn Ile Leu Asn Val Ser Tyr Asn Asn Leu Ala Gly Ala
35 210 215 220

Val Pro Thr Asp Asn Asn Phe Thr Arg Phe Ser His Asp Ser Phe Leu
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Thr Gly His Arg Asp Lys Pro Pro Ile Ser Lys Ala Ala Ile Ile Gly
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Val Ala Val Gly Gly Leu Val Ile Leu Leu Met Ile Leu Val Ala Val
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Cys Arg Pro His His Pro Pro Ala Phe Lys Asp Ala Thr Val Ser Lys
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Ala Leu His Val Phe Asp Asp Ile Met Arg Met Thr Glu Asn Leu Ser
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Glu Lys Tyr Ile Ile Gly Tyr Gly Ala Ser Ser Thr Val Tyr Lys Cys
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Val Leu Lys Asn Cys Lys Pro Val Ala Ile Lys Lys Leu Tyr Ala His
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Tyr Leu Gln Ser Leu Lys Glu Phe Glu Thr Glu Leu Glu Thr Val Gly
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Ser Ile Lys His Arg Asn Leu Val Ser Leu Gln Gly Tyr Ser Leu Ser
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15	Lys Asn Ile Leu Leu Asp Lys Asp Tyr Glu Ala His Leu Thr Asp Phe		
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20	Val Met Gly Thr Ile Gly Tyr Ile Asp Pro Glu Tyr Ala Arg Thr Ser		
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25	Arg Leu Asn Glu Lys Ser Asp Val Tyr Ser Tyr Gly Ile Val Leu Leu		
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30	Glu Leu Leu Thr Gly Lys Lys Pro Val Asp Asn Glu Cys Asn Leu His		
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610 615 620

His Gln Leu Pro Pro Gln Pro Ser Pro Pro Ala Tyr Val Asp Glu Tyr
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Val Ser Leu Arg Gly Thr Gly Ala Leu Ser Cys Ala Asn Ser Ser Ser
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Thr Ser Asp Ala Glu Leu Phe Leu Lys Phe Gly Glu Ala Ile Ser Gln
660 665 670

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Asn Met Val
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU03/00854

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. ⁷: C12Q 001/60; C12N 015/29

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

SEE ELECTRONIC DATABASE BOX BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SEE ELECTRONIC DATABASE BOX BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE AGRICOLA CAPLUS WPIDS: PLANT? ARABIDOPSIS PROTEIN KINASE ERECTA ER
TRANSPIRA? DESSICA? RESPIRAT? LOCUS GENE ALLELE POLYMORPH? GENOTYP?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database accession number AY106598. Zea mays PC006596 mRNA sequence. 1273 bp. 16 October 2002. Hainey CF, Dolan M, Miao GH, Vogel JM, Whitsitt MS, Arthur LW, Hanafey M, Morgante M and Tingey SV. See mRNA sequence (comprises SEQ ID Nos 21, 23-25, 27-29, 31-34, 36-41 and 43).	24-26
X	Rauh BL, Basten C and Buckler IV ES (2002). Quantitative trait loci analysis of growth response to varying nitrogen sources in <i>Arabidopsis thaliana</i> . Theor Appl Genet 104: 743-750. See the entire document.	29, 30
A	Toril KU, Mitsukawa N, Oosumi T, Matsuura Y, Yokoyama R, Whittier RF and Komeda Y (1996). The arabidopsis <i>ERECTA</i> gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. The Plant Cell 8: 735-746. (Cited in the application at page 6.) See the entire document	1-28, 31-35



Further documents are listed in the continuation of Box C



See patent family annex

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search
27 August 2003

Date of mailing of the international search report
- 1 SEP 2003

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INTERNATIONAL SEARCH REPORT

International application No. .

PCT/AU03/00854

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Douglas SJ, Chuck G, Dengler RE, Pelecanda L and Riggs CD (March 2002). <i>KNAT1</i> and <i>ERECTA</i> regulate inflorescence architecture in arabidopsis. The Plant Cell 14: 547-558. See the entire document.	1-28, 31-35